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Attempts to enhance production of pleuromutilin via heterologous expression of the pleuromutilin biosynthesis gene cluster in various host systems

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Abstract

Pleuromutilin and its semisynthetic derivatives show great potential as antibiotics for human use, however production from its natural fungal source gives a relatively low yield. For further development of this drug, an efficient approach for enhancing the production of pleuromutilin is needed. Heterologous expression is a promising approach that allows transfer and expression of the entire pleuromutilin biosynthetic pathway in new hosts. This strategy could solve the limitation of low yield and also may allow the modification of the pathway to make novel products in these new hosts.

Initially *Aspergillus oryzae* was used for heterologous expression of the pleuromutilin biosynthesis pathway. In an attempt to increase flux through the pathway, the *pdc* promoter was chosen to replace the weak *eno* promoter. The *pdc* promoter could effectively drive the *GFP* gene in *A. oryzae*, however transformants containing biosynthetic genes under control of the *pdc* promoter did not show any improvement in accumulation of the desired metabolites and the reasons for this are still unclear. Plant-based production systems may provide an alternative to filamentous fungi, so *Nicotiana tabacum* was explored as a heterologous host. Using the Golden Gate assembly method, expression vectors were developed to express part or all of the seven gene pleuromutilin biosynthesis pathway. Through transient *Agrobacterium* infiltration of leaves, it was demonstrated that candidate pleuromutilin intermediates were likely being produced, however this was correlated with chlorosis in the leaf tissues suggesting a toxic effect *in planta*, limiting this direction of research. Given others have had success using yeast-based expression systems for terpene products, this was also employed as a platform for the heterologous expression of pleuromutilin biosynthesis genes. The pathway-specific GGS and Cyclase were expressed under regulation of the *GAL* promoters and a product consistent with the initial pleuromutilin precursor was detected, however GAL-based induction was not reliable and unexpectedly, yields were not enhanced in the yeast host AH109 which ought to give elevated titres for diterpene products.

Whilst all three host systems proved to be amenable to manipulation, in all cases, further optimisation of gene expression is required in order to obtain the desired levels of enzyme activity needed to improve pleuromutilin production.

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Lastly, there is no word to describe how much my parents have done for me for the past four years. I would like to show my deepest appreciation to them for their unconditional love, understanding and endless support during the long journey of my study.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University’s Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic degree. Except where indicated by specific reference in the text, the work is the candidate’s own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed .  Date.....20/07/2020.....

Suphattra Sangmalee

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List of Abbreviations

°C	Celsius degree
adeA	Phosphoribosylaminoimidazolesuccinocarboxamaide synthase gene
adh	*A. oryzae* alcohol dehydrogenase gene
AmpR	Ampicillin resistance gene
AMR	Antimicrobial resistance
antiSMASH	Antibiotics and secondary metabolites analysis shell
argB	Ornithine transcarbamylase gene
ATF	Acetyl transferase
ATP	Adenosine triphosphate
BAGEL	Bayesian Analysis of Gene Expression Level
bar	Resistance to Basta gene
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CABP	Community-acquired bacterial pneumonia
CDC	The Centers for Disease Control and Prevention
ccdB	*E. coli* toxin gene
cDNA	Complementary DNA
CH_3CN	Acetonitrile
CMP	Czapek-dox broth premix, maltose, peptone medium
CYC	Cyclase
CYP	Cytochrome P450
CZD	Czapek-dox broth premix, sorbitol, agar medium
DMAPP	Dimethylallyl diphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative light scattering detector
eno	*A. oryzae* enolase gene
FDA	Food and Drug Administration
FF	Forward
FPP	Farnesyl-diphosphate
g	Gram

gDNA	Genomic DNA
GFP	Green fluorescent protein
GGPP	Geranyl-geranyl-diphosphate
GGS	Geranyl-geranyl-diphosphate synthase
GN	Glucose, nutrient broth medium
gpdA	*A. nidulans* glyceraldehyde-3'-phosphate dehydrogenase gene
GPP	geranyl diphosphate
GRAS	Generally Regarded as Safe organism
H_2O	Water
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IPP	Isopentenyl diphosphate
IV	Intravenous
kb	Kilobase
KCl	Potassium chloride
$KMnO_4$	potassium permanganate solution
L	Litre
LB	Bactopeptone, yeast extract, NaCl medium
LC-MS	Liquid chromatography-mass spectroscopy
M	Molar
m/z	Mass to charge ratio
MEA	Malt extract, agar medium
MEP	Mevalonate pathway
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MRSA	Methicillin-resistant Staphylococcus aureus
MS	Mass spectrometry
MVA	Mevalonate pathway
N_2	Nitrogen
NaCl	Sodium chloride
NADHP	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
nptII	Kanamycin resistance gene
NRPS	Nonribosomal peptide synthetase
NSAR1	*Aspergillus oryzae* strain (*nia*D-, *s*C-, Δ*arg*B, *ade*A-)
OSMAC	one strain many compounds

P450	Cytochrome P450 monooxygenase
PCR	Polymerase chain reaction
pdcA	Pyruvate decarboxylase
PEG	Polyethylene glycol
pgkA	Phosphoglycerate kinase
PKS	Polyketide synthetase
ptrA	Thiamine thiazole synthase
RiPP	Ribosomally synthesized and post-translationally modified peptides
rpm	Revolutions per minute
RR	Reverse
SAR	Structure-activity relationships
SDR	Short-chain dehydrogenase/reductase
SM	Yeast nitrogen base, ammonium sulphate, glucose, yeast synthetic drop-out medium supplements without uracil, agar medium
SOB	Tryptone, yeast extract, NaCl, KCl, $MgCl_2$ $MgSO_4$ medium
SOC	Tryptone, yeast extract, NaCl, KCl, $MgCl_2$ $MgSO_4$, glucose, medium
SpecR	Spectinomycin resistance gene
TAE	Tris base, acetic acid, ethylenediaminetetraacetic acid
TCM	traditional Chinese medicine
TLC	Thin layer chromatography
TSA	Tryptic soy, agar medium
TSB	Tryptic soy medium
TTC	2,3,5-Triphenyl-2H-tetrazolium chloride
U	Unit
V	Volt
UV	Ultraviolet
v/v	volume/volume
w/v	Mass/volume
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPAD	Yeast extract, peptone, glucose, adenine, agar medium
YPD	Yeast extract, peptone, glucose, adenine medium

Chapter 1 Introduction

1.1 Antibiotics

Antibiotics refer to a group of drugs that have the ability to prevent and treat bacterial infection. Unfortunately, the overuse and misuse of these medicines led to the rapid emergence of resistant bacteria around the world (O'Neill, 2015). This crisis results in a high mortality rate of patients and a great economic burden on the health care system worldwide. This situation also reflects the lack of development for new antibiotics. To overcome this problem, research efforts to discover and develop a new antibacterial agent are urgently needed.

1.1.1 History of antibiotic from its natural source

The majority of the antibiotic drugs that are available in the market originally came from natural sources such as bacteria, fungi and plants. The treatment of infections started several thousand years ago long before people realized that the infections were caused by bacteria. In the pre-antibiotic era, the evidence that fungal and plant extracts were used to treat infections were well-documented in writing from ancient Egypt, Greece, and China. One of the successful treatments was the topical application of filamentous fungi grown on bread to treat infected wounds from the ancient Egyptians (Cowan, 1999, Ventola, 2015). The utilization of this mould benefit continued for many years and was referenced and published in the book called Theatrum Botanicum by John Parkinson in 1640 (Gould, 2016). Another well-known example is qinghaosu (later known as artemisinin), an anti-malaria drug which was extracted from the leaves of *Artemisia annua* plant and used by Chinese herbalists in their traditional Chinese medicine (TCM) as a remedy for many illnesses for thousands of years (Aminov, 2010, Gould, 2016). Moreover, traces of tetracycline have been detected in the bones of the ancient Sudanese Nubian population and the late Roman period during occupation of Egypt hinting at its use, although the origin of the tetracycline is still unknown (Cook et al., 1989).

The modern era of antibiotics came into being with two famous researchers, Paul Ehrlich and Alexander Fleming, who reported a breakthrough of antibiotic discovery to the world. In the late 19th century Paul Ehrlich observed the action of a chemical dye that could stain some bacteria but not the others. He hypothesized that there was a possibility to create a compound

that could selectively kill specific bacteria without being harmful to the others or to the host. Ehrlich's idea of a "magic-bullet" came along with a large scale and systematic screening program in 1904 (Aminov, 2010). He conducted lab work together with the chemist Alfred Bertheim and the bacteriologist Sahachiro Hata, to discover a drug that could treat syphilis, a sexually-transmitted disease that was widespread and incurable at that time, caused by *Treponema pallidium*. During that time syphilis was treated with inorganic mercury salts but the poor results came together with serious side effects. According to their project, hundreds of organoarsenic derivatives of a highly toxic drug Atoxyl were synthesised and tested against rabbits infected by syphilis. Within the 600^{th} series of tested compounds, the compound number 606 showed a strong result, curing infected rabbits and further study of this compound on humans also showed a promising result of treatment by IV injection (Ehrlich and Hata, 1910). In 1909, Salvarsan, the arsenic-based compound which was proved to cure syphilis efficiently, was launched into the market. This drug could be called the first synthetic antibiotic of the modern world (Gould, 2016).

The systematic screening approach by Paul Ehrlich became a standard method in the pharmaceutical industry as a strategy to search for new drugs. Thousands of drugs were synthesised and screened in clinical trials for antibiotic properties. As a result, a sulfa (sulphonamide) drug named Prontosil, was synthesized by Josef Klarer and Fritz Mietzsch in 1932. Its antibiotic activity was tested and it proved to be active against streptococcal infection (Domagk, 1935). Subsequently, a later study demonstrated that Prontosil had no direct antibiotic activity (Welch, 1937). It was claimed to be a prodrug of the sulfa drug. However, the mass production of Prontosil and its derivatives had already been done by many companies, due to its cheap production cost, ease of modification and being off-patent. This phenomenon contributed to one of the most widely disseminated drug resistance cases in the world, the sulfa drug resistance (Aminov, 2010).

The discovery of Penicillin (Figure 1) by Alexander Fleming in 1928 is probably the most well-known case for the discovery of an antibiotic. Penicillin was found after an observation made by Fleming of a *Staphylococcus* bacteria plate he accidentally left on the bench, that had become contaminated by a fungus, *Penicillium notatum* (Durand et al., 2019). He noticed that there was a clear zone free of bacteria, wherever the fungus grew on the plate. So, he concluded that the fungus might secrete a substance which has the ability to kill bacteria. Unfortunately, even after 12 years following this observation, Fleming still faced a problem

with the purification of the active compound from fungal culture. In 1939, Howard Florey and Ernst Chain, together with their team, resolved this issue and successfully worked on the extraction of the fungal culture and obtained the pure compound, which they named as penicillin. The drug had a potent antibiotic activity, largely against Gram-positive bacteria (Nicolaou and Rigol, 2018).

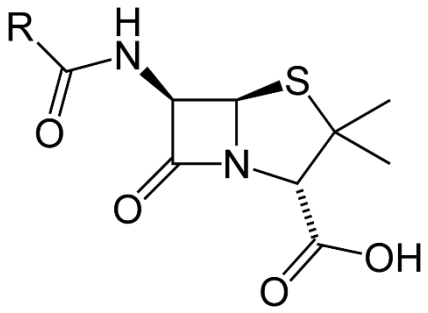


Figure 1 Penicillin structure.

Even though there were a number of synthetic antibiotics which were discovered and isolated prior to penicillin, it was penicillin that made its way to become successful as the first mass produced antibiotic in the world, being used to save many lives of soldiers during the World War II (Gould, 2016, Nicolaou and Rigol, 2018). After the breakthrough of penicillin, many more antibiotics were discovered and isolated, especially during 1950s to1970s which has since become known as the golden age of the discovery of antibiotics.

The pioneer in the discovery of antibiotics in this era was Selman Waksman, an American biochemist. Unlike Fleming who discovered penicillin by accident, Waksman developed a novel systematic way to screen bacteria for the antibiotic activity known as the “Waksman platform”. The basic principles for his screening methods were based on the observation of the growth inhibition zones of the tested strain (strain that was suspected to produce an antibiotic) over the indicator strain (targeted strain). There are three main methods to test on an agar plate; the cross-streak, the spot on the lawn and the well diffusion (see Figure 2) (Durand et al., 2019). These platforms had advantages over the animal screening model by being faster and using less resources, so became widely used for a mass screening of expected antibiotic-producing organisms. Both academics and industry were inspired by the Waksman platform and led to many new classes of antibiotics during the golden era of antibiotic discovery.

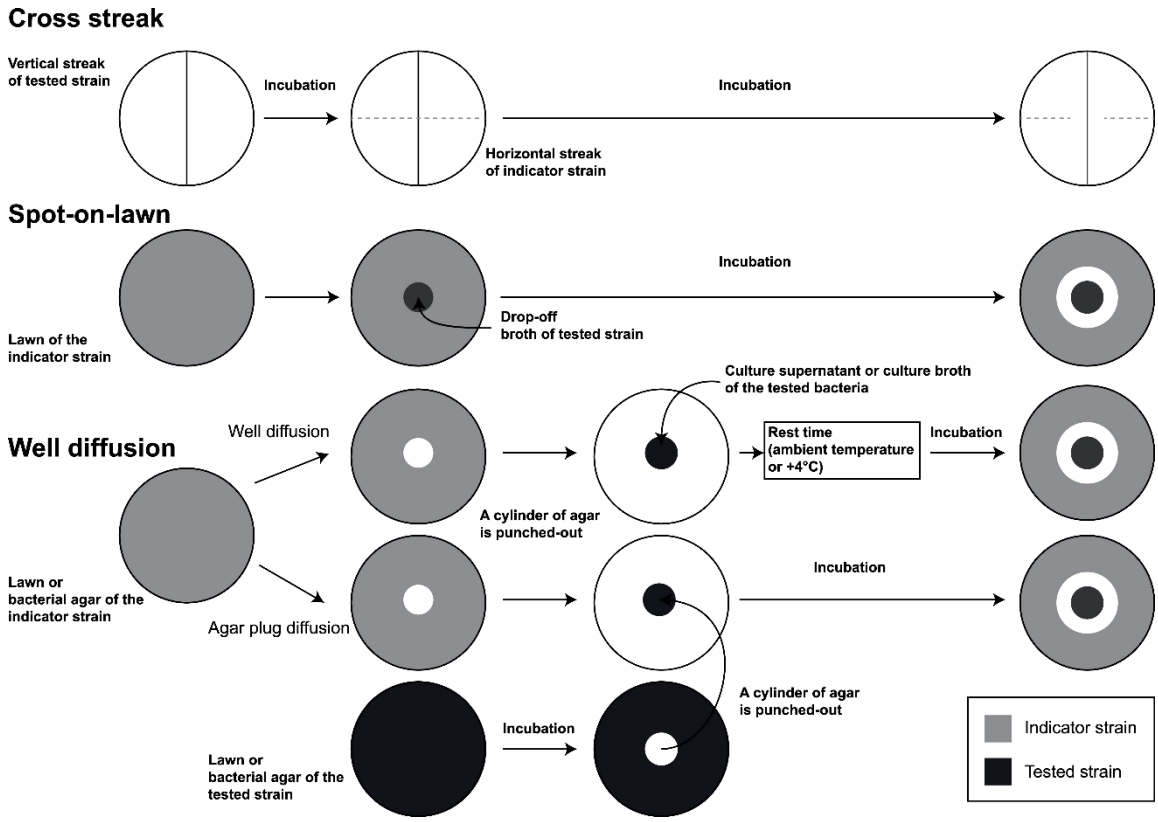


Figure 2 Illustrates the three methods from Waksman platforms; 1) the cross-streak method: by streaking the test strain vertically on agar plate and incubating until it reaches its exponential phase, then a horizontal streak of indicator strain is made, and the plate is incubated again and the inhibition zone is monitored. 2) the spot on the lawn method: screening process is done by adding a single drop of the test strain on a lawn of indicator strain on an agar plate, then after incubation the inhibition zone is observed and 3) the well diffusion method: based on the diffusion of liquid broth or the supernatant of the test strain through an agar plate which is covered with a lawn of an indicator strain and then the clearing zone is measured. (sourced from (Durand et al., 2019).

By using his own screening platform, Waksman successfully discovered around twenty new antibiotics which mostly came from the Actinomycetes, such as streptomycin, neomycin and actinomycin. Streptomycin, an antibiotic produced by the soil organism *Streptomyces griseus*, was discovered by the initial systemic screening of soil bacteria by Waksman and his team in 1944. It was the first antibiotic which was developed after penicillin and was effective in treating tuberculosis (Durand et al., 2019). After the kick-start of streptomycin and successful screening methods from Waksman, many research efforts were made

screening other organisms for the production of antibiotics, but the results were very limited. In 1952, the pharmaceutical company Eli Lilly asked for help from missionaries to send soil samples from various exotic locations for the screening of new antibiotics. Vancomycin was then discovered from *Amycolatopsis orientalis*, a soil sample sent from the jungle in Borneo. It was indicated for use in the treatment of penicillin-resistant *Staphylococcus aureus*. Due to the fast development of penicillin-resistance by staphylococci, the drug was given fast track approval by the FDA and launched into the market under the trade name Vascocin in 1958 (Gould, 2016). However, it was not used as a first line drug, because it had to be given by IV and was later found have toxic effects to ears and kidney. Soon after, a less toxic drug, methicillin was developed and used to treat penicillin-resistant *Staphylococcus aureus*. Methicillin, the first β-lactamase-resistant semi-synthetic penicillin was subsequently developed by Beechams in 1959. But in 1961, only two years after the discovery of methicillin, the first report of *S. aureus* strain resistance to methicillin occurred in United Kingdom and was so called the birth of Methicillin-resistant *Staphylococcus aureus* (MRSA). This soon spread internationally to other European countries and later to Japan, Australia and the United States (Enright et al., 2002). The emergence of methicillin resistant strains caused a sharp increase in hospital acquired infections. MRSA isolates also often showed resistance to other β-lactams drugs or to the entire class of penicillin-like antibiotics including penicillin, amoxicillin, methicillin *etc*. The actual origin of MRSA is still poorly understood. Many studies have been conducted to characterise and identify the relatedness of MRSA isolates from individual hospitals or countries, but no correlation has been found and that makes it more difficult to combat this resistant disease (Hussain et al., 2000).

Aside from the focus on the screening of new organisms for antibiotic production, there is a question of how the growth conditions affect the production of secondary metabolites. Thus, a hypothesis arose based on growing a strain under several different conditions that might lead to a higher yield or production of new antibiotic compounds (Hewage et al., 2014). This issue has been noticed since the early days of the development of penicillin for the industrial mass production (Peláez, 2006).

Microorganisms usually contain various cryptic biosynthetic gene clusters, which are not related to characterized molecule of any secondary metabolites. These biosynthetic gene clusters are thought to be silent, which refer to the gene clusters that are unable to express under normal laboratory conditions. The strategy of one strain many compounds (OSMAC) has been developed and shown as an efficient tool that can activate many silent gene clusters. The concept of this cultivation-based approaches is aimed to investigate how a single strain can produce different secondary metabolites when grown under different culture conditions such as medium composition, temperature, aeration or the addition of chemical elicitors (Bode et al., 2002, Romano et al., 2018). The study by Bode et al., (2002) demonstrated that the fungus *Aspergillus ochraceus*, which was known to produce only the main metabolite aspinonene, was able to produce 15 additional metabolites after changing cultivation parameters such as temperature, salinity, aeration, and even the shape of the flasks.

Another support from the study of the genome of two *Streptomyces* species; *S. coelicolor* and *S. avermitilis* (Bentley et al., 2002, Ikeda et al., 2003). The result from both cases showed that the genome of the bacteria contains the genes involved in the response of external stimuli, various stress conditions and the variety of nutrient sources. Due to lack of knowledge of the enzymes activities within the pathway, lead to the problem of how to stimulate the full function of the metabolic potential of the microorganisms under the laboratory conditions and then no new drugs were discovered. In addition, the general strategy was done by assigning a different growth condition with random factors, resulted in the production of known compounds or useless metabolites with lacking a desired antibiotic property. In order to solve this problem, further investigation of the genomics, bioinformatics and microbial physiology together with its mode of action is needed (Peláez, 2006).

1.1.2 Chemical synthesis and semi-synthetic of antibiotic

Since the 1970s, the discovery rate of novel antibiotics declined then completely stalled. This coincided with widespread use of antibiotics, caused a rapid growing resistance problem. With few, if any, new compounds in the discovery pipeline, focus moved towards a modification strategy of the existing core structures, the so called semi-synthetic route. The objectives of this strategy were to enhance the antibiotic activity, broaden the spectrum of activity and minimize or overcome any resistance mechanisms, alongside reduced human toxicity (Aminov, 2010).

Typically, each group of antibiotics shares the same core structure or scaffold. During the semi-synthetic procedure, the core structure is maintained because this is usually responsible for molecule connectivity and key structure-activity relationships (SAR). In order to improve pharmaceutical properties of the drug, new chemical groups may be introduced into the periphery of the molecule (Fischbach and Walsh, 2009). For example, Erythromycin was first of the Macrolide group, discovered from soil samples. It is a metabolite of *Saccharopolyspora erythraea*. Due to low yields and structurally complexity of the erythromycin molecule, a total synthesis was unrealistic, so the second generation such as clarithromycin were developed via a semisynthetic route and this contains a methoxy group at the C6 position of the lactone ring (Figure 3). As a result, clarithromycin has improved acid stability when compared to the first-generation macrolide, erythromycin. When the macrolide resistance emerged, telithromycin, a ketolide and a third-generation macrolide was developed. Ketolide is an erythromycin derivative characterized by replacing the cladinose sugar with keto-group at C3 and introducing carbamate extension into the lactone macrocycle at C11/C12. The modification to telithromycin demonstrated a greater binding affinity of the drug to its target site, and also improved its microbiological properties to be active against the bacteria resistant to erythromycin (Douthwaite, 2001, Mabe et al., 2004).

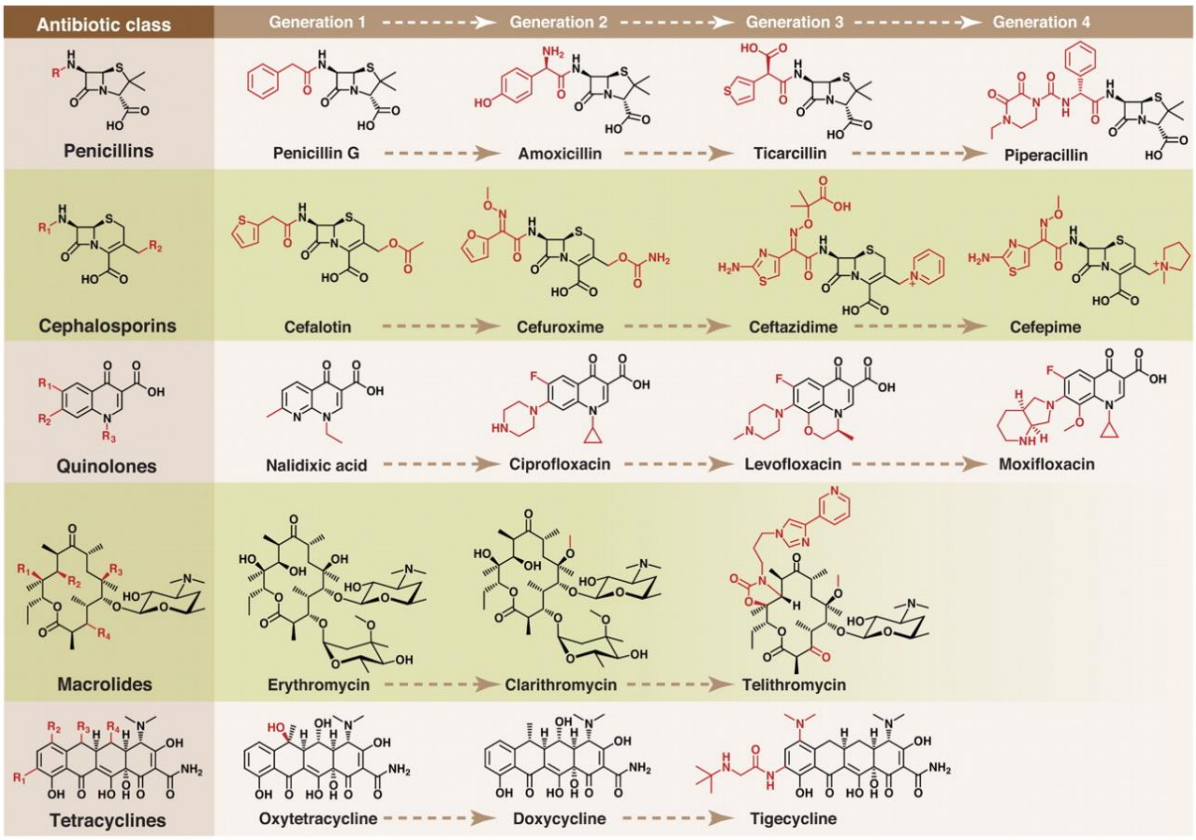


Figure 3 The development of next generation antibiotics through a chemical modification of their original scaffolds. Source from (Fischbach and Walsh, 2009).

This strategy for improving the existing scaffolds was very successful and has continued to provide new effective antibiotics for the market but very few truly new antibiotics have been developed in the last twenty years. It has highlighted that resistance to each new variant does occur, sometimes very rapidly, and shows that there is an ongoing challenge to continue to manage bacterial infections (Aminov, 2010).

1.1.3 The antibiotic resistance crisis

Antibiotic resistance refers to the ability of bacteria to resist the action of the antibiotic that targets them (Blair et al., 2015). During the evolution of resistance, bacteria can acquire a pre-existing resistance gene via other bacteria or from DNA in the environment. It is also reported that exposure to sub-lethal doses of antibiotic can result in selection of mutations in bacteria, resulting in increased resistance to the drug through modifications to outer membranes or other mechanisms that result in reduced permeability to the drug (Munita and Arias, 2016).

There are three major groups of mechanisms that can describe this action. Firstly, limiting the entry of the antibiotic by reducing permeability of cell membrane, or increasing action of the efflux transporters will cause a poor penetration and minimize the intracellular concentration of antibiotic in the bacteria cell. Secondly, mutation of the target site can alter the efficiency of drug-binding so reducing the drugs effect. Thirdly, the bacteria may produce an enzyme that can make changes to the antibiotic molecule. Such a direct modification of the antibiotic structure may result in the antibiotic losing its ability before it reaches the target site (Blair et al., 2015).

Antibiotic or antimicrobial resistance (AMR) was named as one of the top three global health crises of the 21st century by the World Health Organization (WHO) (WHO, 2015). A rapid emergence of AMR is leading to greater effects in terms of morbidity, mortality and economy impact for any population and government worldwide. The Centres for Disease Control and Prevention (CDC) provided a report showing that in the United States more than 2 million people are affected by organisms that are resistant to antibiotics each year and at least 23,000 died. In European countries, according to a current report, 25,000 people died as a result of infection by an antibiotic-resistant organism annually (CDC, 2019). Moreover, the report also highlighted a significant increase in mortality caused by what were regarded as comparatively manageable diseases such as *Klebsiella pneumoniae* within the European populations during the past four years. Some predictions are that the number of such deaths globally will reach nearly 300 million by 2050 and it is estimated that the global economic loss of up to $100 trillion is caused by antibiotic resistance (O'Neill, 2015).

There are several factors that have made the antibiotic resistance problem become worse. The biggest cause is the overuse of antibiotics. In some countries, antibiotics are an over the

counter drug, which patients can easily access without a prescription. Studies showed a statistically significant correlation between the increase consumption of antibiotics and the increase of antibiotic resistance (Goossens et al., 2005, Baquero et al., 2002). Another major issue is a lack of new antibiotics launched in the market. A lack of economic appeal due to a low cost of antibiotic when compared with the drugs for treatment of chronic diseases, has led many pharmaceutical companies to give up on antibiotic development. Besides this, the difficulties in pursuing regulatory approval are always an obstacle and may have led to the reduction in the number of new antibiotics in the market (Ventola, 2015).

1.1.4 A current approach for treating bacterial infection

After the golden age of antibiotics, most pharmaceutical companies have reduced their efforts in the discovery and development of antibiotics or completely withdrawn from this process. Antibiotic discovery is a time-consuming process of screening and charactering for active compound and gives a poor return on investment compared to development of other drugs (Durand et al., 2019). A traditional strategy for natural compound discovery is defined as a top-down process. It starts with finding and collecting specimens from diverse environments. This is followed by cultivating under laboratory conditions, extraction and then antibiotic activity screening and characterizing for a desired compound. All these techniques use a complicated system to elucidate new active compounds without any knowledge involved in the biosynthesis and often result in re-isolation of known compounds (Luo et al., 2014). As a result, there is a big gap in the antibiotic pipeline to release a new drug.

In contrast, the bottom-up approach for novel natural product discovery has focussed on genome sequence data. This uses various gene manipulation methods to identify the genes and enzymes involved in the biosynthesis pathway of interest. These approaches have provided a powerful strategy to uncover hidden gene clusters from microorganisms and may allow the production of a metabolite from normally silent gene clusters in the host organisms that may have the potential to be a new antibiotic drug (Luo et al., 2014). Several bottom-up approaches have been created and utilized recently and will be described as follows.

1.1.4.1 Genome mining approach

Genome mining is a revolutionary approach of the procedure to discover secondary metabolites from natural sources. It refers to the process of finding a potential secondary metabolite based on genome sequence information (Bachmann et al., 2014, Choi et al., 2018). There are many bioinformatic tools available and widely used for mining procedure such as BLAST, HMMer, BAGEL, PRISM and anti-SMASH. These tools are employed for selecting a target biosynthetic gene cluster for two main purposes. Firstly, to identify core genes from the known scaffolds. This strategy (Figure 4) based upon the homology comparison with the known secondary metabolite gene clusters, from the conserved motifs for each pathway type; polyketides synthetase PKS, nonribosomal peptide synthetase (NRPS) or ribosomally synthesized and post-translationally modified peptides (RiPP). A new metabolite with any small novel variation in its structure may cause a significant change of the biological activity of product (Durand et al., 2019, Ward and Allenby, 2018).

Secondly comes identifying tailoring enzyme genes within the target biosynthetic gene cluster, that are responsible for modification of the core structure and that may also have an impact on its biological activity. The method is more challenging in terms of defining the extent for the whole biosynthetic gene cluster (Choi et al., 2018, Ward and Allenby, 2018). However, the major problem of genome mining approach for discovery of new antibiotics is that this approach usually requires for the expression of the whole biosynthetic gene cluster from native host or in a heterologous host (Durand et al., 2019).

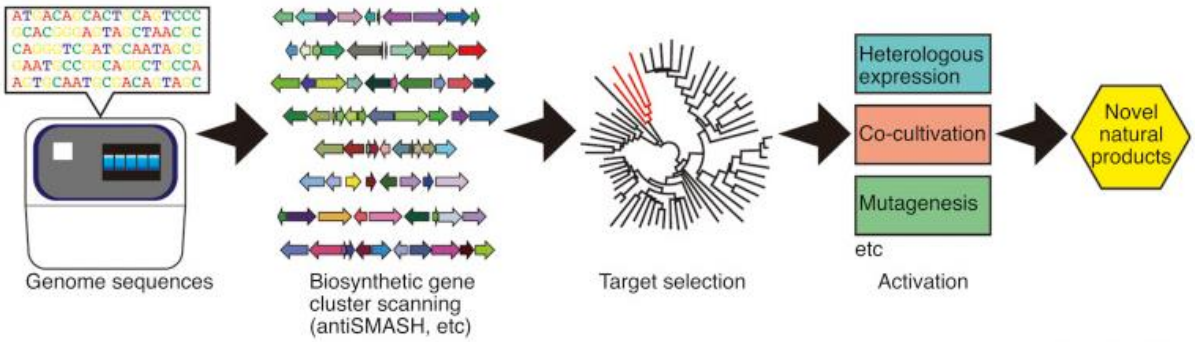


Figure 4 A typical genome mining scheme. Microbial genome data obtained from genome sequencing using a next generation genome sequencer, followed by phylogenetic analysis of a targeted biosynthetic gene cluster using bioinformatic tools such as anti-SMASH. Then each cluster will be analyzed for its function. After the selection of a putative biosynthetic gene cluster some methods will be provided in order to awake a silent gene cluster. Finally, a potential product is uncovered. Source from (Choi et al., 2018).

1.1.4.2 Biological gene cluster activation approach

The cluster activation/stimulation approach was developed in order to solve some difficulties that arise from genome mining. Under typical laboratory conditions many biosynthetic gene clusters are silent or barely expressed so their products would not be readily apparent. Therefore various stimulating approaches such as optimization of regulatory network, use of chemical elicitors, co-cultivation and heterologous expression have been developed in order to awake some sleeping biosynthetic gene clusters and drive that cluster from genome mining into reality (Choi et al., 2018).

For example, the optimization of regulatory networks using a strong promoter to activate a gene cluster was successfully applied to Large ATP binding of the LuxR (LAL) pathway-specific regulator, a silent type I polyketide biosynthetic gene cluster of *Streptomyces ambofaciens* (Laureti et al., 2011). The overexpression of LAL under the control of a strong constitutive *ermE** promoter, resulted in the production of stambomycins A-D, four glycosylated macrolides as a product from the gene cluster. Another example comes from a chemical elicitor method, which showed that addition of atenolol in liquid culture of *Streptomyces hiroshimensis* led to the discovery of two novel antibiotics, taylorflavin A and

taylorflavin B (Moon et al., 2019). The most widely applicable method for biosynthetic gene cluster stimulating is heterologous expression, the concept is to clone a target cluster and transform it into a host in a way that guarantees its expression. The principal of this method will be discussed later in the section **1.3**.

1.1.5 A metabolic model for a yield improvement - the penicillin case study

The process of penicillin discovery, the subsequent strain improvement and its large-scale production was a great breakthrough both for the pharmaceutical industry and for the therapy of infectious diseases (Ligon, 2004).

The study published by Raper et al., (1944), reported the screening of related fungal isolates mostly from *P. notatum* and *P. chrysogenum* for the production of penicillin on agar plates. However, they noted that the area of inhibition zone and the production of penicillin were not directly related. Later, Moyer and Coghill (1946) reported that the production of penicillin from the isolate of *P. chrysogenum* was more suitable than Fleming's original *P. notatum*. Initially, Moyer and Coghill (1946) could successfully increase the production of penicillin by focusing on the culturing conditions of the fungus in liquid culture. They found that using lactose as a carbon source, as well as the supplement of corn steep liquor into the fermentation medium could lead to a ten-fold increase of penicillin production.

Besides the screening of strains and the optimization of culturing conditions, a random mutagenesis and consequent screening was employed to generate new mutant strains. This approach was widely used for strain improvement for industrial scale production. The theory is based on the exposure of the desired strains, which were already known to provide a high titre for the production of penicillin, with concentrated chemical agents such as nitroso-methyl guanidine or strong physical agents like X-ray or UV radiation. This caused random genetic mutations in the treated strains and was then followed by screening for increased titre (Rowlands, 1984). In the case of *P. chrysogenum* NRRL 1951 (wild type) that was treated with X-ray, this provided the X-1621 mutant strain which increased the yield up to 300 μg/mL of penicillin. The Q-176 strain was then obtained from several rounds of classical mutagenesis and produced up to 550 μg/mL of penicillin (Jami et al., 2010). The Q-176 strain was also known as the ancestor of the Wisconsin mutant strains; as named Wis 54-1255, which is a laboratory model strain. The Wis 54-1255 strain then generated many lines of high

producer mutant strains for penicillin included the overproducer mutant strains that are currently used for the industrial production of penicillin, which could yield up to 50,000 μg/mL in fed batch culture (Jami et al., 2010). Subsequent analysis of the mutant strains generated through consecutive rounds of mutagenesis showed that this approach led to the duplication of the gene cluster of penicillin biosynthesis. Although there was a direct correlation between increased copy number and the increase of titre of penicillin, a plateau effect occurred when the copy number of gene cluster reached 5 copies (Newbert et al., 1997). This suggests that there might be other limiting factors that affect the titre of antibiotic production for instance changes to the other primary or secondary pathways within the same strain could alter the rate limiting precursor of penicillin (Newbert et al., 1997).

Proteome studies (Jami et al., 2010) were conducted to observe and compare the cytosolic-proteomes of three penicillin producing strains; *P. chrysogenum* NRRL 1951 (wild type strain), *P. chrysogenum* Wisconsin 54-1255 (a moderate penicillin mutant strain) and *P. chrysogenum* AS-P-78 (a high producer mutant strain). Among the three different strains, the improved high-producer AS-P-78 strain contains five to six copies of the penicillin biosynthesis gene cluster agreeing with the previous report from (Newbert et al., 1997). In addition, this strain also contains abundant microbodies, the organelles involved in the final step of penicillin pathway, when compared to the other strains. The result also showed that only the wild type strain contains a protein involved in pigment formation. Overrepresented proteins involved in secondary metabolism pathways of terpenoids and porphyrins were also found in the wild type, while the improved strains contain the lower amount of such proteins involved in other secondary metabolite pathways. Hence, it could be hypothesised that a very low penicillin yield of the wild type resulted from the interference of the other secondary pathways which could decrease the metabolic flux through the penicillin biosynthesis pathway. The overexpression of cysteine synthase, the enzyme that converts serine to cysteine, an amino acid essential to penicillin biosynthesis pathway, was detected in the Wis 54-1255 strain when compared to wild type. The high producer strain AS-P-78 also contains a high level of cysteine synthase with additional increase in level of cystathionine *β*-synthase, the enzyme that converts methionine to cysteine. This proved that the high producer strain AS-P-78 over-expresses two cysteine biosynthesis pathway genes which could increase flux through the penicillin pathway compared to the other two strains. Finally, the results from the high producer strain AS-P-78 also indicated a high level of protein involved in the formation of NADPH, the redox cofactor required for the biosynthesis of cysteine. With the increase in

cysteine biosynthesis of the high producer strain AS-P-78, this may in part explain why this strain provides the highest yield of penicillin among the three strains (Jami et al., 2010).

The effective enhancing of penicillin production is an excellent example of how the low titre fungal secondary metabolite can be increased. The results from the intensive work on penicillin production have pointed out several factors to improve the yield of the antibiotic. Small increase in titre by the addition of copy number, but much of the increase is likely to be blocking or reducing the divert of metabolic flux cause by the other secondary metabolite pathways. The development of penicillin can also be a guideline to suggest the possibility for the improvement in the production of pleuromutilin from its natural source, *Clitopilus passeckerianus*, the basidiomycete fungus which is the main goal of this PhD thesis.

Generally speaking, when considering the time and cost for the development of a new drug starting from the basic research study from its natural source until it make its way to the mass production and then to market, the whole process will have taken around 12 years or more (Figure 5) with the investment cost around £1.15 billion (DiMasi et al., 2010). Previous studies showed that for every 10,000-25,000 compounds that are screened and studied in the laboratory, only around 250 make it to the preclinical trial stage and then 5 might make their way to the clinical trial in humans, then finally perhaps only one can get approved by FDA and released into the market (Torjesen, 2015). This points out that a large number of candidate agents with reasonable yield will need to be discovered and produced before finding a likely usable product. Hence, it is worth looking for an alternative approach in order to shorten the time and also improve the yield for the production of a new drug. Heterologous expression systems should be considered as an option to address the limitation of the desired organism that has difficulty growing or is unable to grow under normal laboratory conditions. The use of heterologous expression in a new host system may help to solve the issue of the very low to non-existant expression of the genes within the biosynthetic pathway in the native host and then the improvement and scalable production for a desired secondary metabolite may become more tractable (Harvey et al., 2018).

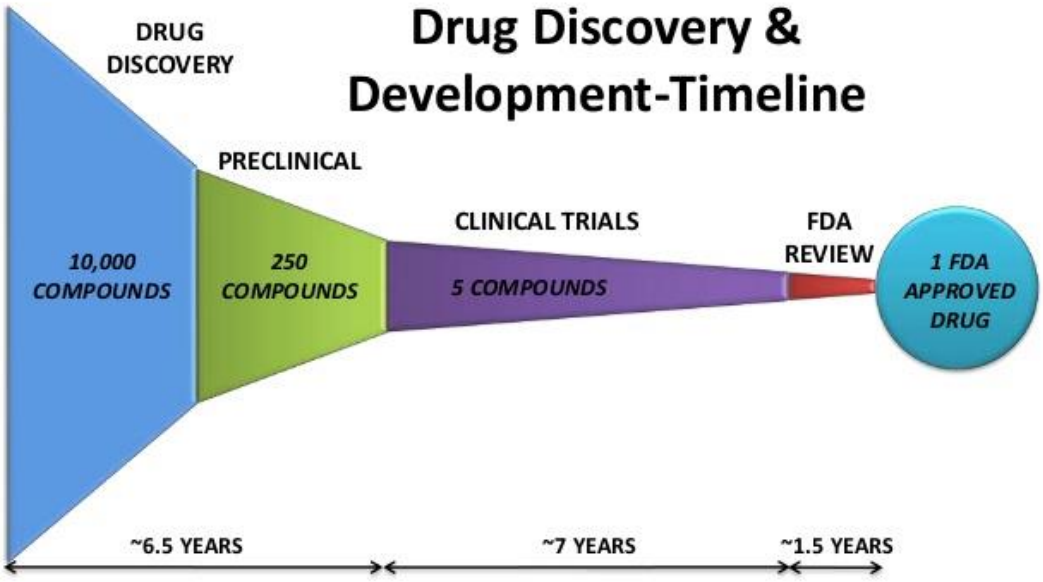


Figure 5 This shows the stages involved in the development of new drugs and the approximate time required for each stage. Image from (Markolin, 2017).

1.2 Pleuromutilin

1.2.1 Development of pleuromutilin and its derivatives

With no new antibiotics being discovered, there has been an interest in revisiting compounds that were discovered during the golden age of antibiotics but that were not further developed at that time; one such compound is pleuromutilin. Pleuromutilin (Figure 6) was discovered and isolated in a crystalline form from two species of Basidiomycete fungi, *Pleurotus mutilus* (now, *Clitopilus scyphoides*) and *P. passeckerianus* (now, *Clitopilus passeckerianus*), by (Kavanagh et al., 1951). Pleuromutilin is a tricyclic diterpenoid antibiotic with antibacterial activities against Gram-positive pathogens and mycoplasma (Ling et al., 2014). In the 1960s, the structure of this antibiotic was elucidated and reported by both Arigoni's group (Arigoni, 1962) and Birch's group (Birch et al., 1966) as a rather rigid 5-6-8 tricyclic carbon skeleton with eight stereocentres (Egger and Reinshagen, 1976, Shang et al., 2013).

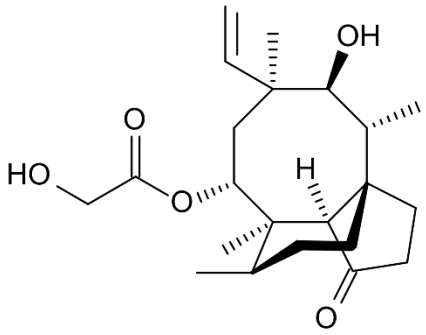


Figure 6 Pleuromutilin.

Previous studies have shown that this antibiotic has a unique mode of action, which involves inhibition of protein synthesis by selectively binding to the peptidyl transferase centre of the 50S bacterial ribosome subunit but has no effect on eukaryotic protein synthesis and does not bind to the mammalian ribosome (Hirokawa et al., 2008, Poulsen et al., 2001). Thus, cross-resistance to other marketed antibacterial agents rarely occurs. The novel mode of action of pleuromutilin made it an attractive target in the development of novel antibiotics for the treatment of multi-drug resistant bacterial infections (Liu et al., 2015).

Since the 1960s, extensive effort has been made to develop new pleuromutilin derivatives (Figure 7) with more potent antibacterial activity for human use (Ling et al., 2014). As a

result, two compounds have been successfully developed, but both of them were used as therapeutic agents for veterinary purposes. Tiamulin, the first practical pleuromutilin derivative, was used as a prophylactic and therapeutic agent against anaerobic bacteria, intestinal spirochetes and *Mycoplasma spp*. in swine and poultry (Xu et al., 2009). While, Valnemulin was approved in 1999 by the European Union and was used in treatment of enzootic pneumonia of pigs (Drews et al., 1975). Although both Tiamulin and Valnemulin were successfully applied in veterinary medicine, due to their rapid metabolism they were not suitable for human use. From then on, several other semisynthetic compounds were designed and developed for human use. These efforts resulted in the development of Azamulin, which entered phase I clinical trials in the 1980s, but no further progress was made due to its low solubility in water and short half-life (Hildebrandt et al., 1982). In 2007, Retapamulin, the first derivative of pleuromutilin was approved by Food and Drug Administration (FDA) for human use as a topical antimicrobial agent to treat skin infections (Wang et al., 2012).

More recently, several others pleuromutilin derivatives BC-3781, BC-3205 and BC-7013 have been developed for human use. All of them have already entered phase I clinical studies. Most notably amongst these, BC- 3781 (known as, Lefamulin) has successfully completed phase II clinical trials (Rubino et al., 2015) and is now in stage III trials as an orally available product (Mendes et al., 2016). It has excellent antimicrobial activity against relevant bacteria, including MRSA (Ling et al., 2012, Shang et al., 2013). The results demonstrated a therapeutic potential of BC-3781 for the treatment of skin infections and bacterial lung infections, including community-acquired bacterial pneumonia (CABP), and other indications.

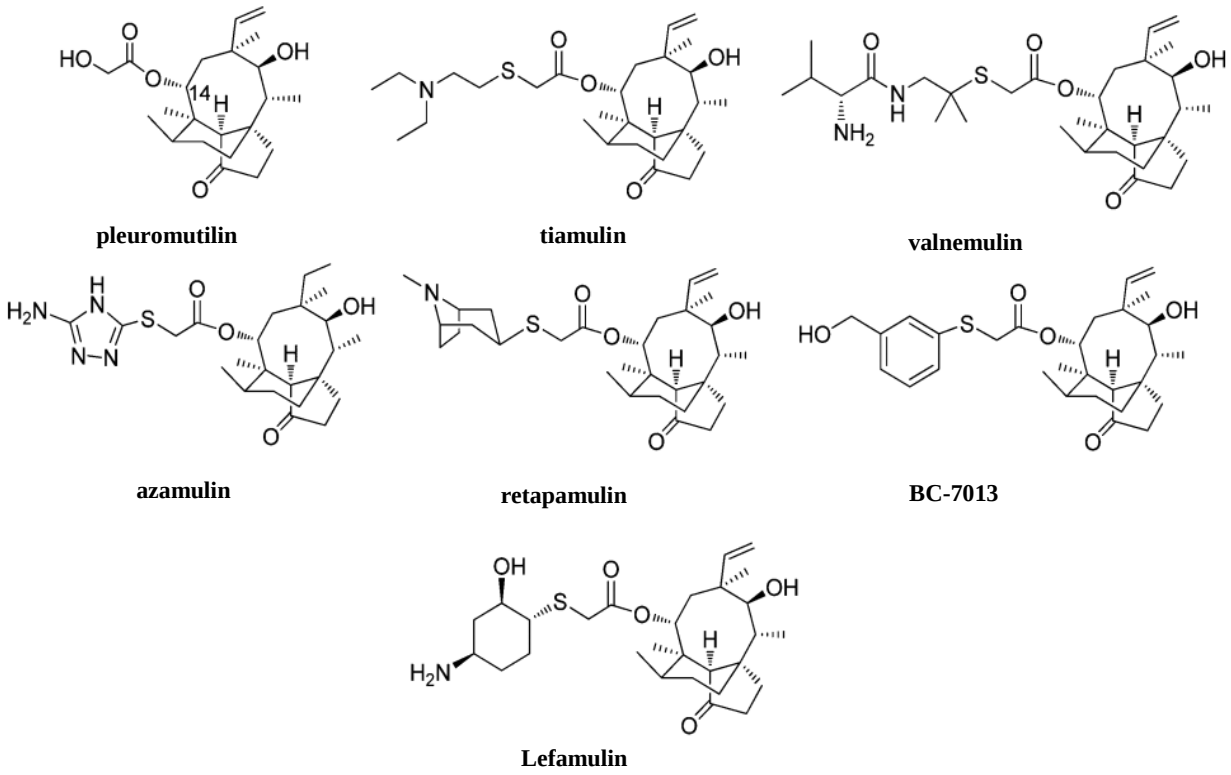


Figure 7 Chemical structures of pleuromutilin derivatives showing the various synthetic side chains at the C14 position.

1.2.2 Pleuromutilin biosynthesis pathway and gene cluster

Pleuromutilin is a diterpene isolated from the basidiomycete fungi *C. scyphoides* and *C. passeckerianus*. It belongs to the class of secondary metabolites known as terpenes, which are produced in fungi through the mevalonate pathway (MEP pathway) (Lange et al., 2000).

The Basidiomycota have proven to be a valuable group for production of various terpenoid compounds. Terpenoids are compounds that originated from the basic of the five carbon units dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). In fungi, the mevalonate pathway uses acetyl-CoA as substrate and converts it two isoprene units, DMAPP and IPP. Sequentially, the two isoprene units are condensed by prenyldiphosphate synthase enzymes in a head-to-tail manner to yield a linear hydrocarbon of varying lengths: C10 geranyl pyrophosphate (GPP), C15 farnesyl pyrophosphate (FPP), and C20

geranylgeranyl pyrophosphate (GGPP) (Quin et al., 2014, Wawrzyn et al., 2012). Further coupling of these hydrocarbons via dephosphorylation and cyclization by terpene synthases/ cyclase enzymes can provide the more complex terpenes. Fungal terpene synthases are known to have a potential to generate sesquiterpenes (C15), diterpenes (C20) and triterpenes (C30) based on the length of the precursor molecules. Moreover, enzymes such as cytochrome P450 monooxygenases, oxidoreductases, and various transferases are involved in the modification of the terpene scaffold in biosynthetic pathways, to gain the final terpenoid compound (Quin et al., 2014).

Fungi often have many different secondary metabolic pathways and the genes for each are typically located in clusters within fungal genomes (Walton, 2000). This feature provides the advantage for natural product discovery and bioengineering in that it facilitates genome mining approaches in the identification of biosynthetic pathways. Once a gene within a secondary metabolic pathway was identified, there is a high possibility that other genes in the pathway will be closely associated and therefore relatively easily identified by bioinformatic methods (Quin et al., 2013, Wawrzyn et al., 2012).

The genomic study of pleuromutilin biosynthetic gene cluster in *C. passeckerianus* was done to identify the key genes encoding the production of pleuromutilin in a cluster within the same genomic region. According to the study, seven genes have been isolated and sequenced as the genes responsible for pleuromutilin production shown in Figure 8 (Bailey et al., 2016).

Figure 8 Biosynthetic gene cluster for pleuromutilin biosynthesis from *C. passeckerianus*.

The bottleneck enzymes that control the production of pleuromutilin biosynthetic gene cluster (Figure 9) are predicted to be Geranyl-geranyl diphosphate synthase (GGS) and Cyclase (CYC). GGS is responsible for supplying geranylgeranyl diphosphate (GGPP) to the pathway. GGPP is a general precursor of diterpenes. CYC triggers the formation of tricyclic structure of pleuromutilin via the cyclisation of GGPP. In addition, there are three cytochrome P450s (P450-1, P450-2 and P450-3) involved in the pleuromutilin biosynthetic gene cluster. The P450-1 and P450-2 play a critical role to modify the core structure of the hydrocarbon skeleton of pleuromutilin by adding hydroxyl groups to C-11 and C-3. The short-chain dehydrogenase/reductase enzyme (SDR), the tailoring enzyme within the cluster converts the C-3 hydroxyl group to ketone group. Acetyl-transferase (ATF) adds the acetyl group to the C-14 OH of mutilin, giving 14-acetyl-mutilin. Finally, P450-3 reduces the acetyl side-group of the scaffold resulting in the complete structure of pleuromutilin (Alberti et al., 2017, Bailey et al., 2016). The pathway has been fully elucidated by heterologous expression in *Aspergillus oryzae*, however titres were low.

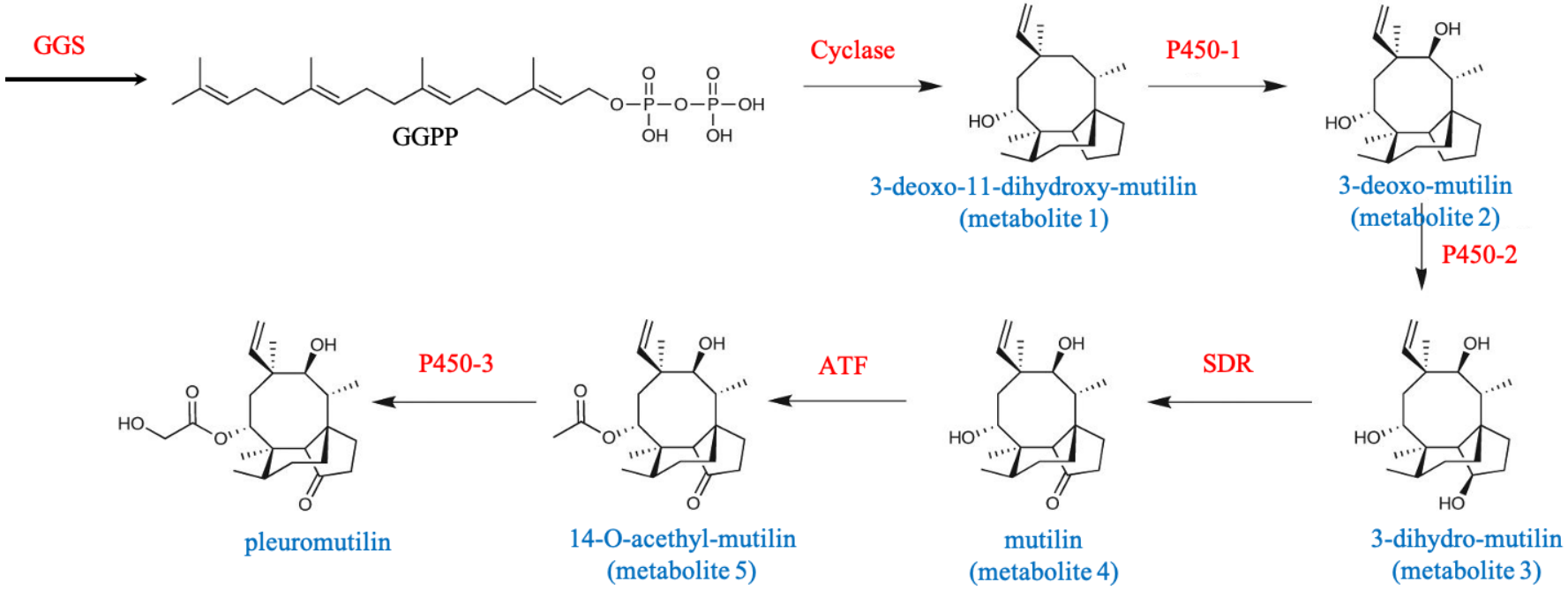


Figure 9 Proposed biosynthetic pathway of pleuromutilin.

1.3 Heterologous expression system

With the main purpose of my thesis to enhance the production yield of pleuromutilin, heterologous expression of the pleuromutilin biosynthesis pathway in different host systems will be considered as a rational avenue to improve the pleuromutilin production. Due to the very low titres of pleuromutilin produced by the original fungus, *Clitopilus passeckerianus,* an alternative system for pleuromutilin production on a large scale is required. The basic principle of heterologous expression involves the identification of genes corresponding in biosynthetic pathway and transfer those genes to a different host for synthesis of the compound. A critical step for this procedure is the selection of the expression system in which pros and cons of several factors have to be considered (Gomes et al., 2016). Thus, there are several choices of systems that could be an alternative host for the heterologous expression of pleuromutilin as follows.

1.3.1 Heterologous expression in bacteria

The first and most commonly used host is the bacteria *Escherichia coli* (*E. coli*). Due to its simplicity, rapid growth under well-defined laboratory conditions on an inexpensive medium, high efficiency to accept a foreign DNA and protein expression at a high levels, and most of the available cloning and expression vectors are compatible with *E. coli,* it remains the first choice for heterologous expression (Kaur et al., 2018).

However, in spite of many advantages there are also limitations. *E. coli* is unable to perform post-translational modifications which are often required for functional activity after protein synthesis. Protein degradation by proteases enzymes from the host cell can degrade the protein of interest. It has an inefficient secretion system to transport and release protein into media. The expressed enzyme may produce a potentially toxic product and thus kill the host (Kaur et al., 2018, Makrides, 1996). Most importantly for this project, the *E. coli* system is not appropriate for making a broad-spectrum antibiotic that is active against gram negative bacteria, which makes it unsuitable for heterologous expression of pleuromutilin biosynthetic gene cluster.

1.3.2 *Aspergillus oryzae* as secondary host system for heterologous expression

Aspergillus oryzae (A. oryzae) is a filamentous ascomycete fungus from the family Trichocomaceae. It has been widely used as a genetic model for studying a range of biological processes. Several studies have shown the potential of *A. oryzae*, as a prolific producer of secondary metabolites (Ehrlich and Mack, 2014, Keller et al., 2005).

In this study, *A. oryzae* was chosen as a secondary host system for heterologous expression of pleuromutilin gene cluster by taking its two major advantages. Firstly, it is a well characterized organism that has a long history in the Japanese food and fermentation industry for food production such as sake, miso and soy sauce (Feng et al., 2004). It also has been recognized as a Generally Regarded as Safe (GRAS) organism, which means that it does not produce any toxins or other dangerous metabolites and therefore is safe to be used for the production of enzymes and other molecules intended for human use (Barbesgaard et al., 1992).

Secondly, its ability to secrete high level of metabolites (Lubertozzi and Keasling, 2009), as well as its well-established culturing methodology and development of genetic manipulation tools including several selectable markers, which are essential to rescue transformed colonies, have been validated for transforming this fungus. Furthermore, the development of improved promoter systems from *Aspergillus spp*. is expected to ensure a higher yield of heterologous expression of foreign genes (Pahirulzaman et al., 2012, Yamada et al., 1997).

1.3.3 Using yeast for heterologous expression

The yeast *Saccharomyces cerevisiae* is a heterologous expression system that has been widely used for the production of many bioactive compounds. This system has several attractive characteristics to be used as a tool for the production of foreign metabolites including; easy and rapid growth under basic laboratory conditions, inexpensive culturing

conditions, high biomass and scalable fermentation, an ability for post-translational modification and secretion systems, safety and wide use in the food industry, easily accessible with the advanced molecular genetic tools (Baghban et al., 2019). However, the

disadvantages of this host system are low yields, cell stress due to the presence of the foreign genes and lack of a strong inducible promoter (Gellissen et al., 2005).

Alternative yeast system such as *Pichia pastoris*, *Hansenula polymorpha*, *Arxula adeninivorans* have been developed, each system also exhibits different pros and cons (Mokdad-Gargouri et al., 2012). For instance, *Pichia pastoris* offers stronger promoters to drive gene expression and higher ability of protein modification, *Hansenula polymorpha* and *Arxula adeninivorans* can assimilate nitrate and use a wide range of carbon sources (Gellissen et al., 2005). The different capability of each system provides an option for a heterologous expression host in yeast. From a range of these candidates, the selection for the most suitable expression platform will give a desirable targeted product.

1.3.4 Expression of heterologous genes in plant systems

Over the past decade, the utilization of plant-based expression systems for the production of secondary metabolites and recombinant compounds has gained attention. Many studies have proven that the system could be an interesting alternative platform to produce important recombinant proteins such as antibodies, vaccines and therapeutic pharmaceuticals (Ghag et al., 2016). Several applications in plant biotechnology are aimed at the large-scale production of natural secondary metabolites. As a molecular platform, plants also offer significant advantages over the traditional expression systems like prokaryotic or eukaryotic microbes, including lack of animal pathogens, low production costs and easy scalability (Fischer et al., 2004). Furthermore, dried leaves and seeds containing a recombinant protein can be stored at room temperature for long periods of time without loss of its properties (Kolotilin et al., 2014).

When considering the secondary metabolites from plants, there are three main categories based on their biosynthetic pathways: phenolic compounds, nitrogen-containing compounds and terpenes. Focusing on terpenes, the commercial drugs such as artemisinin, taxol, and vincristine are all biological products of terpenoids that originated from plants. In common with other organisms, terpenoid biosynthesis in plants starts with the universal precursor of terpenoids, IPP. However, unlike *Saccharomyces cerevisiae* and *Escherichia coli*, plants have two pathways responsible for IPP biogenesis; a cytosolic mevalonate (MVA) pathway and a plastidic 2-methyl-erythritol 4-phosphate (MEP) pathway (Ikram et al., 2015). The two pathways are a well-established strategy to enhance the amount of carbon going into

dedicated isoprenoid precursors, pushing metabolic flux through to the final biologically active metabolite (Ikram et al., 2015). This makes plants an attractive host system for heterologous expression of the pleuromutilin gene cluster.

The tobacco plant, *N. tabacum* has a long history as a successful host plant in molecular biology and therefore the strongest candidate for the production of secondary metabolite. The major advantages of this plant over others include a well-established technology for gene transfer and expression, high biomass yields, high seed number and year-round growth and harvesting (Makhzoum et al., 2014). Furthermore, tobacco reduces risk of transgenic material either in feed crops or the human food chain because it is a non-food or non-feed crop (Twyman et al., 2003).

Heterologous expression of one or a few genes in plants to produce secondary metabolites has been successfully presented in several studies. One such example is the alteration of steroid compounds using heterologous expression of one animal gene, *CYP*11A1, a cDNA of cytochrome P450scc from the bovine adrenal cortex, in transgenic tobacco. The analysis result showed that the expression of this heterologous gene caused an up-regulation and formation of steroid compounds such as pregnenolone and progesterone in tobacco plants (Spivak et al., 2010). Another example explored the heterologous expression of five artemisinin biosynthetic genes from *Artemisia annua* in *Nicotiana tabacum* resulting in the production of artemisinin precursors, amorphadiene (Zhang et al., 2011), so it may be possible to express the seven genes of the pleuromutilin pathway *in planta*.

1.4 Summary

It is without doubt that natural products have been the main source of antibiotic drugs. Their discoveries also inspired their derivatives for many novel antibiotics available today. However, the discovery and development of antibiotic has been held back due to the length of the lead time and high cost of the research, development and clinical trial processes and regulatory barriers. This might not have been an issue until the emergence of antibiotic resistance and its impact on public health around the world. This phenomenon awakes an urgent calling to renew the effort in the discovery and development of new antibiotics, especially as there are very few new products in the pipeline. But thanks to advances in technology, creation of new molecular methods could open the way forward to discover the new antibiotic of the future.

1.5 Aims and objectives

The aim of my PhD project is to understand and improve the biosynthesis of pleuromutilin and its production. In order to achieve this, I will express the pleuromutilin gene cluster and recreate the biosynthetic pathway in three different host systems; *Aspergillus oryzae*, *Saccharomyces cerevisiae* and *Nicotiana tabacum*, all of which have already been widely used in other studies as hosts for the production of biologically active substances.

There are various objectives that I am aiming to achieve during this project:

- To create improved expression vectors by replacing or improving the promoters involved in the expression of the genes responsible for the biosynthesis of pleuromutilin in the secondary host *A. oryzae*, through heterologous expression of the gene cluster with the aim of enhancing the production of the antibiotic in this secondary host

- To build up the full seven genes of the pleuromutilin biosynthetic pathway in a suitable vector for heterologous expression in *N. tabacum*

- To construct the yeast expression vector containing the genes responsible for the biosynthesis of pleuromutilin in *S. cerevisiae*

- To assess the expression of the genes inserted and evaluate chemical analysis of transformed *A. oryzae* strains, transformed yeast and transgenic plants, then compare metabolites production from the three different hosts

Chapter 2. Materials and Methods

Materials and Methods

All chemicals and media used within this project were analytical grade or molecular grade, and were purchased from Fisher Scientific, Formedium, Melford, Oxoid, Sigma-Aldrich, Thermo Scientific, or VWR.

Deionised water was used to prepare all media and solutions. A standard sterilisation procedure by autoclaving at 121^{o}C for 15 minutes was applied to all media, glassware, plasticware and solutions before use.

2.1 Microbial strain, growth media and storage condition

All microbial strains used in this project were grown and maintained in a different media and growth conditions as shown in **Table 1**

Table 1 List of microbial strains used in this project.

Organism	Strain	Media for growth maintenance
Aspergillus oryzae	NSAR1 (*ΔargB, adeA−, niaD−, sC−*)	MEA+4 at 28^{o}C
Saccharomyces cerevisiae	BY4742 (Y10000) (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*)	YPAD at 28^{o}C
Saccharomyces cerevisiae	YPH499 (*MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*)	YPAD at 28^{o}C
Saccharomyces cerevisiae	AM109 (*Mat a/α, PGal1-HMG2(K6R):: HOX2, ura3, trp1, his3, PTDH3-HMG2(K6R)X2-::leu2 ERG9/erg9, UBC7/ubc7, SSM4/ssm4, PHO86/pho86, derived from AM102*)	YPAD at 28^{o}C
Bacillus subtilis	ATCC 6633	TSB at 28^{o}C
Escherichia coli	One shot® ccdB Survival™ 2 T1^{R}	LB at 37^{o}C
Agrobacterium tumefaciens	LBA4404	LB at 28^{o}C

Table 2 Microbial media components.

Media	Components
MEA+4	15 g/L malt extract, 1.5 g/L methionine, 2 g/L ammonium sulphate, 0.1 g/L adenine, 1 g/L arginine / 15 g/L agar
YPAD	10 g/L yeast extract, 20 g/L bactopeptone, 20 g/L D-glucose, 40 mg/L adenine hemisulfate / 15 g/L agar
LB	10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract / 15 g/L agar
TSB	30 g/L tryptic soy broth / 15 g/L agar

Table 3 List of antibiotics used in this project. A stock solution was prepared, then calculated and supplied into media to reach the final concentration of working concentration.

Antibiotic	Solvent	Stock solution concentration	Working solution concentration
Ampicillin	Water	100 mg/mL	100 µg/mL
Kanamycin	Water	50 mg/mL	50 µg/mL
Spectinomycin	Water	50 mg/mL	250 µg/mL
Rifampicin	Methanol	20 mg/mL	20 µg/mL

For short-term storage, all fungal, yeast and bacterial cultures were stored on agar plates with appropriate medium and supplement at 4^{o}C.

For long-term storage, a plug of growing mycelia of fungal strain, including transformant strains were stored by adding sterilised glycerol (40% v/v in water) and quickly frozen in liquid nitrogen before storage at -80^{o}C. Yeast and bacterial liquid cultures were added to an equal volume of sterilised glycerol (40% v/v in water) and rapidly frozen in liquid nitrogen then stored at -80^{o}C.

2.2 Molecular methods

2.2.1 Plasmid DNA purification

Plasmid DNA from *E. coli* was purified by using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer's instructions. In brief, the overnight culture was pelleted and treated with an alkaline lysis to release the plasmid DNA. The cleared lysate was then neutralised to precipitate the cellular debris while maintaining the plasmid DNA. The supernatant containing the plasmid DNA was loaded into a spin column and the plasmid DNA was absorbed onto a silica membrane. After several washing, the impurities were washed away, and the purified plasmid DNA was then eluted with a small volume of elution buffer.

Plasmid DNA from yeast was purified by using the Zymoprep TM Yeast Plasmid Miniprep I (Zymo Research) according the manufacturer's instruction. Firstly, yeast cells from an overnight culture were collected and resuspended in digestion buffer. Zymolase enzyme was added to disrupt the yeast cell wall and produce protoplast. The mixture was incubated for 1 hour at 37^{o}C. The lysate mixture was then treated with lysis and neutralising buffer to precipitate the cell debris. The supernatant containing the plasmid DNA was then rescued by isopropanol precipitation. Finally, the pelleted plasmid DNA was resuspended with small volume of TE buffer.

2.2.2 Small-scale fungal genomic DNA purification

Small scale fungal DNA purification was employed as described by (Liu et al., 2000). The protocol started by picking up a 1 mm^3 clump of mycelia from the agar plate and putting into 1.5 mL Eppendorf tube containing 500 µL of lysis buffer (400 mM Tris-HCl [pH 8.0]), 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulphate). The mycelia were then disrupted with a sterile loop and left at room temperature for 10 min. After that 150 µL of potassium acetate solution (60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of distilled water, pH 4.8) was added, then briefly vortexed, and centrifuged at 10,000 x g for 1 min. The supernatant was transferred to a new tube and centrifuged at 10,000 x g for 1 min once again to remove all precipitate. After transferring the supernatant to a new tube, an equal volume of isopropanol was added and briefly mixed by inversion, then

centrifugation at 10,000 x g for 2 min. The supernatant was discarded and 300 µL of 70% ethanol was used to wash the pellets, centrifuged at 10,000 x g for 1 min and the supernatant was discarded. The DNA pellet was air dried and dissolved in 50 µL of TE buffer (10 mM Tris-HCl. 1 mM EDTA, pH 8.0) before storing at -20^{o}C.

2.2.3 Restriction digestion of DNA

Restriction digestion was done on the plasmid vector to obtain a linear backbone prior proceeding with yeast-based homologous recombination. It was also carried out for screening and confirming the identity of the correct construction of the plasmid after *E. coli* plasmid DNA purification. All restriction enzymes were purchased from Thermo Fisher Scientific and restriction digestion reaction was performed by following the manufacture's protocols.

The restriction digestion mixture was set up with 1 µg of plasmid DNA, 1 µL of 10X FastDigest Green Buffer, 1 µL FastDigest enzyme and made up to the final volume of 10 µL with sterile water. The mixture was incubated for 30 minutes – 1 hour at 37^{o}C. Unless specified, the components of restriction digestion mixture were combined as indicated.

2.2.4 PCR

2.2.4.1 Conventional PCR

In order to amplify cDNA fragments, check ligation reactions, confirm transformant strains and for colony screening, conventional PCR reactions were carried out using DreamTaq Green DNA Polymerase (Thermo Scientific). A 2X DreamTaq Green Mix was prepared as follows: 2 mL of 10X DreamTaq Green Buffer, 400 µL of 10 mM dNTPs, 100 µL of DNA Polymerase (5U/ µl), then made up to 10 mL with sterile deionised water. The aliquot of 500 µL 2X DreamTaq Green Mix was prepared in 1.5 mL Eppendorf tube and stored at -20^{o}C.

When needed the PCR reactions were set up with the following components; 10 µL of 2X DreamTaq Green Mix, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 1 µL (1-10 ng) of template DNA and the 20 µL final volume was made up by sterile water.

Cycling parameters for conventional PCR were initial denaturation at 95°C for 3 minutes, then 35 cycles of denaturation at 95°C for 30 seconds, annealing at "$T_{annealing}$" for 30 seconds

and extension at 72°C for 1min/1kb (depending on the length of the expected PCR product), then followed by a 10 min final extension at 72°C. The PCR reactions were carried out by using a PTC-200 DNA Engine Thermal Cycler (Biorad).

2.2.4.2 Colony PCR

Colony PCR was performed in order to detect the presence of insert DNA in plasmid constructs from *E. coli* transformant colonies growing on selective plates after transformations with plasmids, using 2X DreamTaq Green Mix. Instead of the template DNA, half a colony was picked up from the plate and placed into a PCR tube. The same cycles as for common PCR were followed, however the initial denaturation step at 95°C was extended to 10 minutes. The other half colony was used for further analysis when the PCR reaction had amplified a band of the expected size.

2.2.4.3 High-fidelity PCR

When PCR products needed to be cloned into expression vectors, Phusion High-fidelity DNA Polymerase (Thermo Scientific) was used to amplify the fragment, the reaction mixture was prepared as follows: 0.2 µL Phusion High-fidelity DNA Polymerase (2U/µL), 4 µL 5X Phusion HF buffer. 0.4 µL 10 mM dNTPs, 1 µL 10 µM forward primer, 1 µL 10 µM reverse primer, 1 µL template DNA (1-10 ng) and then sterile water was used to made up the final volume to 20 µL.

Cycling parameters for high-fidelity PCR were; initial denaturation at 98°C for 30 seconds, then 35 cycles of denaturation at 98°C for 10 seconds, annealing at "$T_{annealing}$" for 30 seconds and extension at 72°C for 20 sec/1kb (depending on the length of the expected PCR product), then followed by a 10 min final extension at 72°C. The PCR reactions were carried out by using a PTC-200 DNA Engine Thermal Cycler (BioRad).

2.2.5 Primers

All primers used in this study were purchased from IDT (Integrated DNA Technologies). Each primer was diluted with sterile water at 1:10 ratio to give a concentration of 10 µM and stored at -20ºC.

Table 4 List of primers.

Primer	Sequence (5´-3´)	$T_{melting}$ (ºC)	Description	Product size (bp)
ArgB FF *	CCGTT ACTAGTGGAT CCGAGCTCGG TACCA TCAGTTGGGCCTCATTTG	69.5	Amplification of *ArgB* to be used in yeast homologous recombinant	1498
ArgB RR *	CTGTAATAC GACTCACTAT AGGGAATATT A CATACCCGTCAACTCATCAG	64.2		
ptrA FF (1) *	ATTACATGATGCGGCCCTCTAGATGCATGC TGGGGATCTGACAGACGGGC	71.7	Amplification of *ptrA* to be used in yeast homologous recombinant	737
ptrA RR (1)	TCCTCGCCCTTGCTCACCAT GTTTCAAGTTGCAATGACTATCATCTGTTA	67.9		
prtA/GFP FF (2)	TAACAGATGATAGTCATTGCAACTTGAAAC ATGGTGAGCAAGGGCGAGGA	67.9	Amplification of *GFP* to be combined with *ptrA* in yeast homologous recombinant	720
ptrA/GFP RR (2) *	CGCCAGTGTGCTGGAATTCTGCAGATATCCTT ACTTGTACAGCTCGTCCA	69.1		
pgkA FF (1) *	ATTACATGATGCGGCCCTCTAGATGCATGC ATCGACTAAGTGCCATGGAT	69.4	Amplification of *pgkA* to be used in yeast homologous recombinant	500
pgkA RR (1)	TCCTCGCCCTTGCTCACCAT TATGGACGATGTTCTATCACACAAGGTGGG	70.0		
pgkA/GFP FF (2)	CCCACCTT GTGTGATAGA ACATCGTCCA TA ATGGTGAGCAAGGGCGAGGA	70.0	Amplification of *GFP* to be combined with *pgkA* in yeast homologous recombinant	720
pgkA/GFP RR (2) *	CGCCAGTGTGCTGGAATTCTGCAGATATCC TTACTTGTACAGCTCGTCCA	69.1		
pdcA FF (1) *	ATTACATGATGCGGCCCTCTAGATGCATGC TGACTGTCACGCCAGCGACT	71.7	Amplification of *pdcA* to be used in yeast homologous recombinant	500
pdcA RR (1)	TCCTCGCCCTTGCTCACCAT TGTA AGGACTCAGT AAGAGGAATG GAAAAT	68.3		

Primers	Sequence (5´-3´)	$T_{melting}$ (ºC)	Description	Product size (bp)
pdcA/GFP FF (2)	ATTTTC CATTCCTCTT ACTGAGTCCT TACA ATGGTGAGCAAGGGCGAGGA	68.3	Amplification of *GFP* to be combined with *pdcA* in yeast homologous recombinant	720
pdcA/GFP RR (2) *	CGCCAGTGTGCTGGAATTCTGCAGATATCC TTACTTGTACAGCTCGTCCA	65.0		
pdcA-CYC FF*	ATGACCCACTGGGGTTTTAGGAGGTCAATT TGACTGTCACGCCAGCGACT	71.0	Amplification of *pdcA* to be combined with *Cyclase* in yeast homologous recombinant	500
pdcA-CYC RR*	GCGTGCATGAAGATCTTCAGATAGACCCAT TGTAAGGACTCAGTAAGAGG	66.3		
pdcA-P450-3 FF*	ATGACCCACTGGGGTTTTAGGAGGTCAATT TGACTGTCACGCCAGCGACT	71.0	Amplification of *pdcA* to be combined with *P450-3* in yeast homologous recombinant	500
pdcA-P450-3 RR*	TGGTAGAGCACGTTCCGTTGACGGAGCCAT TGTAAGGACTCAGTAAGAGG	69.5		
pdcA-SDR FF*	ATGACCCACTGGGGTTTTAGGAGGTCAATT TGACTGTCACGCCAGCGACT	71.0	Amplification of *pdcA* to be combined with *SDR* in yeast homologous recombinant	500
pdcA-SDR RR*	GCCTGTGACGATTGCGACCTTGCCTTCCAT TGTAAGGACTCAGTAAGAGG	69.4		
GGS FF F1 *	TAGAGCGGATGTGGGGGGAGGGCGTGAAGA C AA A ATGAGAATACCTAACGTCTT TCTCT	70.4	Amplification of *GGS* fragment 1 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	281
GGS RR F1 **	ATCAATTAGTAGGGATGCATTATGAAGGTCCT CAATGGCCTTTGT TACGC	66.4		
GGS FF F2 **	GGCCATTGAGGACCTTCATAATGCATCCCTAC TAA TTGATGACATCGAAGATGAGTCTGC	68.1	Amplification of *GGS* fragment 2 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	195
GGS RR F2 **	GCAACCTGTGTGCCACCTGTTTTCATGCCAA GGTCATAGA CGTCTTGAAG	69.3		
GGS FF F3 **	TGGCATGAAAACAGGTGGCACACAGGTTGC CAACGCAATGGCTCGCATCT	73.0	Amplification of *GGS* fragment 3 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	580
GGS RR F3 *	ACGACTCACTATAGGGAATATTAAGGAAGAC AACTACTCTGCGATGTACAACTTTTCC	65.9		

Primers	Sequence (5´-3´)	$T_{melting}$ (ºC)	Description	Product size (bp)
Cyclase FF F1 *	TAGAGCGGATGTGGGGGGAGGGCGTGAAGA CAAAATGGGTCTATCTGAAGATCTTCATG	70.5	Amplification of *Cyclase* fragment 1 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	156
Cyclase RR F1 **	GTTTGCCGTCGATCTCACGGGATACCATGGCT ACCCAAGCAGTGTCGTAG	70.7		
Cyclase FF F2 **	GTAGCCATGGTATCCCGTGAGATCGACGGCA AACAAGTCTTTGTCTTTCCAGAGACATTC	69.3	Amplification of *Cyclase* fragment 2 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	804
Cyclase RR F2 **	CAAGAGCATCAGGGTTTTCGAGCTCATGTCA GCGTCGTGAGTGGTGCCGG	72.0		
Cyclase FF F3 **	GACATGAGCTCGAAAACCCTGATGCTCTTGC AAGTTCTCAACCACCCATA	68.8	Amplification of *Cyclase* fragment 3 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	300
Cyclase RR F3 **	GTGCTCGAAAAGGAGACGGACAAGCGCCTG TGAGGACAGCATAGAGGAGT	71.4		
Cyclase FF F4	CAGGCGCTTGTCCGTCTCCTTTTCGAGCACG GAAAGGGCAACCTTAAATC	70.7	Amplification of *Cyclase* fragment 4 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	1120
Cyclase RR F4 **	GAGAGCCTCGTTCTTCATTGATCTGATGACC AAGGAGACAAAGGGCAAAGACGAGGGCGA	71.1		
Cyclase FF F5	GTCATCAGATCAATGAAGAACGAGGCTCTCG CGATTTGGTGGACGTTTTC	68.0	Amplification of *Cyclase* fragment 5 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	500
Cyclase RR F5 *	ACGACTCACTATAGGGAATATTAAGGAAGAC AATCAATGGTGGATTCCATTGCTCCCG	67.6		
P450-1 FF F1	TAGAGCGGATGTGG GGGGAGGGCGTGAAGACAA AATGCTGTCCG TCGACCTCCC GTCTG	74.3	Amplification of *P450-1* fragment 1 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	344
P450-1 RR F1	TATTACCCTG CTTGTCAAGA ATAGTCCGAA CAGCCTTTGG ACTGTTCAGA	67.4		
P450-1 FF F2	TTCGGACTAT TCTTGACAAG CAGGGTAATA TCACAGGAGA TCGGCCATTT TCGCC	69.0	Amplification of *P450-1* fragment 2 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	160
P450-1 RR F2	CTTGGCGTTA GGTAATTGTG GATACCTTTG CGACCAGTTT TCCATACGGA AGTGT	68.6		

Primers	Sequence (5´-3´)	$T_{melting}$ (ºC)	Description	Product size (bp)
P450-1 FF F3	CAAAGGTATC CACAATTACC TAACGCCAAG TGCCTTGAGT GGCTACATAC	67.2	Amplification of *P450-1* fragment 3 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	330
P450-1 RR F3	TCCACAATGG TTTTCCAGCC GGCCCCGGGG AAACCGCGAG GGATGTAGTC	74.5		
P450-1 FF F4	CCCCGGGGCC GGCTGGAAAA CCATTGTGGA TGAATTCAAG GATTTCCGTA	71.7	Amplification of *P450-1* fragment 4 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	240
P450-1 RR F4	AGAATACACG ATTCGATGTA CGATATCGTG GTTTTCGCGC CGGCGTCCAA	70.0		
P450-1 FF F5	CACGATATCG TACATCGAAT CGTGTATTCT TGCTCTTATC GCCCACCCGA	67.0	Amplification of *P450-1* fragment 5 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	395
P450-1 RR F5	ACAGG AAATCGGGAT TGACATCTTC AGATAAGCCT GGCTTCGTGC CGTTT	69.6		
P450-1 FF F6	AGGCTTATCT GAAGATGTCA ATCCCGATTT CCTGTTCGGT GCTGGACGTA	68.8	Amplification of *P450-1* fragment 6 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	130
P450-1 RR F6	GTAAAAATTG AATGCCCAAC AGAGCCTCAT GATGAATAGA CCAGTTGATC GTTTT	66.3		
P450-1 FF F7	ATGAGGCTCT GTTGGGCATT CAATTTTTAC CCAGATTCTT CAAACAAGGA	66.8	Amplification of *P450-1* fragment 7 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	192
P450-1 RR F7	CT ACAACGCAGC GAACGCTTCC TTAATCAGAT CTTCCTTCAT CTTATCTCGA GGT	67.7		
P450-1 FF F8	ATGAGGCTCT GTTGGGCATT CAATTTTTAC CCAGATTCTT CAAACAAGGA	66.8	Amplification of *P450-1* fragment 8 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	225
P450-1 RR F8	AC GACTCACTAT AGGGAATATT AAG GAAGAC AA CT ACAACGCAGC GAACGCTTCC TTA	67.9		
P450-3 FF F1	TAGA GCGGATGTGG GGGGAGGGCG T GAAGAC AA A ATGGCTCCGT CAACGGAACG TGCTC	74.0	Amplification of *P450-3* fragment 1 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	1164
P450-3 RR F1	GAGATGGTTT GATGAGGTAC GCCCATAGGA GTCACAGGCC TCCAGCGGTA	71.2		

Primers	Sequence (5´-3´)	$T_{melting}$ (ºC)	Description	Product size (bp)
P450-3 FF F2	TCCTATGGGC GTACCTCATC AAACCATCTC AGATGACGTT TACAGGGAAT	68.0	Amplification of *P450-3* fragment 2 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	160
P450-3 RR F2	TTCGGTCAAG TATCGCTCAG GCCGGAATTC GTCTGGCTGG GGGTAATCGG TTTCGTCGTT	72.9		
P450-3 FF F3	CTGAGCGATA CTTGACCGAA GATGGTAAGC CTAACAAGGC TGTCAGAGAT CCCTTTGATA	68.8	Amplification of *P450-3* fragment 3 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	362
P450-3 RR F3	AC GACTCACTAT AGGGAATATT AAG GAAGAC AA CTAGCCACT AGCAGGCTTC GTGAAC	67.6		
ATF FF F1	TAGA GCGGATGTGG GGGGAGGGCG T GAAGAC AA A ATGAAGCCCT TCTCACCAGA ACTTC	72.1	Amplification of *ATF* fragment 1 to be use in yeast homologous recombinant	1201
ATF RR F1	AC GACTCACTAT AGGGAATATT AAG GAAGAC AA CTAC TGTGCTACAC GAGGGGGATT C	67.5		
SDR FF F1	TAGA GCGGATGTGG GGGGAGGGCG T GAAGAC AA A ATGGAAGGCA AGGTCGCAAT CGTCA	73.6	Amplification of *SDR* fragment 1 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	344
SDR RR F1	TGACTCTGTC CCATACATCG TCCTCGAAGG TCAACGCGGT CTGGAAGTAG	70.0		
SDR FF F2	CCTTCGAGGA CGATGTATGG GACAGAGTCA TCGATGTCAA CCTGGCTGCA	70.5	Amplification of *SDR* fragment 2 and introduce internal mutagenesis sites to be use in yeast homologous recombinant	515
SDR RR F2	AC GACTCACTAT AGGGAATATT AAG GAAGAC AA CT AAATGACACT CCACCCGTTA TCG	66.4		
L0 GGS FF ***	GG GAAGAC GC CTCA AATG ATGAGAATAC CTAACGTCTT TCTCTCTTAC CTGCGACAAG TC	68.7	Amplification of L0-*GGS* fragment introduced *Bpi*I and fusion sites for module L0 assembly	1089
L0 GGS RR ***	GG GAAGAC GC CTCG AAGC CTA CTCTGCGATG TACAACTTTT CCAAGAATCG TTCAAGACC	69.9		

Primers	Sequence (5´-3´)	$T_{melting}$ (°C)	Description	Product size (bp)
L0 Cyclase FF ***	GG GAAGAC GC CTCA AATG ATGGGTCTAT CTGAAGATCT TCATGCACGC GCCCGAACCC TC	71.9	Amplification of L0-*Cyclase* fragment introduced *Bpi*I and fusion sites for module L0 assembly	2916
L0 Cyclase RR ***	GG GAAGAC GC CTCG AAGC TC AATGGTGGAT TCCATTGCTC CCGTTTGCTG TGACCTTGAT	72.3		
L0 Kanamycin FF ***	GG GAAGAC GC CTCA AATG ATGGGGATTGAACAAGATGGATTGCACGCAG GTTCTCCGGCC	71.2	Amplification of L0-*KanR* fragment introduced *Bpi*I and fusion sites for module L0 assembly	834
L0 Kanamycin RR ***	GG GAAGAC GC CTCG AAGC TCAGAAGAACTCGTCAAGAAGGCGATAGAA GGCGATGCGCTG	73.6		
L0 P450-1 FF ***	GG GAAGAC GC CTCA AATG ATGCTGTCCG TCGACCTCCC GTCTGTTGCG AACTTGGATC CC	73.1	Amplification of L0-*P450-1* fragment introduced *Bpi*I and fusion sites for module L0 assembly	1608
L0 P450-1 RR ***	GG GAAGAC GC CTCG AAGC CT ACAACGCAGC GAACGCTTCC TTAATCAGGT CTTCCTTCAT	71.8		
L0 P450-2 FF ***	GG GAAGAC GC CTCA AATG ATGAATCTTTCTGCTCTGAAGGCTGCTCTGCT TGACAGCAAC	70.5	Amplification of L0-*P450-2* fragment introduced *Bpi*I and fusion sites for module L0 assembly	1614
L0 P450-2 RR ***	GG GAAGAC GC CTCG AAGC CTAATAGTCTGCAACATCGTGGATCACCTGC ACAACTGACTG	71.1		
L0 P450-3 FF ***	GG GAAGAC GC CTCA AATG ATGGCTCCGTCAACGGAACGTGCTCTACCAG TCCTTGTAATA	71.0	Amplification of L0-*P450-3* fragment introduced *Bpi*I and fusion sites for module L0 assembly	1605
L0 P450-3 RR ***	GG GAAGAC GC CTCG AAGC CTAGCCACTAGCAGGCTTCGTGAACGTCAAC GGGCAAGCACG	74.3		

Primers	Sequence (5´-3´)	$T_{melting}$ (ºC)	Description	Product size (bp)
L0 ATF FF ***	GG GAAGAC GC CTCA AATG ATGAAGCCCT TCTCACCAGA ACTTCTGGTT CTATCTTTCA TT	68.9	Amplification of L0-*ATF* fragment introduced *Bpi*I and fusion sites for module L0 assembly	1170
L0 ATF RR ***	GG GAAGAC GC CTCG AAGC CTAC TGTGCTACAC GAGGGGGATT CCATTCCCCC CGGTACAG	73.8		
L0 SDR FF ***	GG GAAGAC GC CTCA AATG ATGGAAGGCA AGGTCGCAAT CGTCACAGGC GCATCCAATG GC	73.3	Amplification of L0-*SDR* fragment introduced *Bpi*I and fusion sites for module L0 assembly	798
L0 SDR RR ***	GG GAAGAC GC CTCG AAGC CT AAATGACACT CCACCCGTTA TCGACCGGAA TAACGACGCC	72.1		
pESC-TRP GGS FF *	ATTCGAATTCAACCCTCACTAAAGGGCGGCC ATGAGAATACCTAACGTCTTTCTCTCTTA	70.3	Amplification of *GGS* to be used in yeast homologous recombinant	1114
pESC-TRP GGS RR *	TTATTTAGAAGTGTCAACAACGTATCTACC CTACTCTGCGATGTACAACTTTTCC	65.1		
pESC-TRP CYC FF *	CTCTATACTTTAACGTCAAGGAGAAAAAAC ATGGGTCTATCTGAAGATCTTCATGCACGC	66.3	Amplification of *Cyclase* to be use in yeast homologous recombinant	2940
pESC-TRP CYC RR *	CTAACTCCTTCCTTTTCGGTTAGAGCGGAT TCAATGGTGGATTCCATTGCTCCCG	69.2		

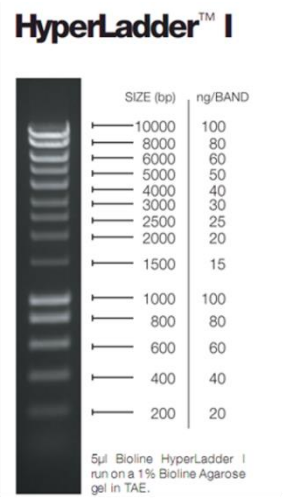
* Primers used to amplify target genes for yeast homologous recombination. The sequences highlighted in **red** are the flanking sequences which are homologous to the sequence of the plasmid backbone.

** Internal primers used to amplify target genes for yeast homologous recombination. *Bpi*I or *Bsa*I recognition sites were found in the genes and needed to be removed, so the internal primers were designed to contain a single mismatch nucleotide highlighted in **red** in order to make a silent mutation but not change the encoded amino acids.

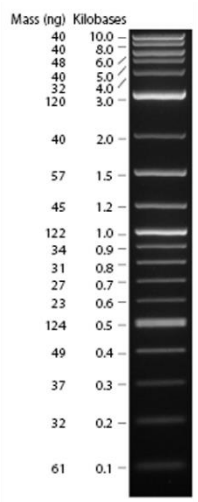
*** Primers used to amplify target genes for Golden Gate assembly level 0. The sequences highlighted in red are *Bpi*I recognition sequences that were introduced in the sequence to generate the 4 nucleotide overhangs after digestion. The sequences highlighted in **blue** are the compatible sequence overhangs that will be combined with the L0 destination vector after ligation.

2.2.6 Gel electrophoresis

To visualise RNA, genomic or plasmid DNA after nucleic acid isolations, as well as for PCR amplification products and restriction digestion fragments, gel electrophoresis was performed on 1% w/v agarose gel. The gel was prepared by dissolving a desired amount of agarose (Type I, Molecular Biology Grade, Bioline) in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) mix with Midori Greene Advance Nucleic Acid Stain. The agarose/buffer mixture was melted by heating in a microwave until the agarose has completely dissolved. The agarose was cooled prior to pouring into a gel caster. Gels were run for 30 min at 100 V for separation in horizontal chamber containing 1X TAE buffer. Five µl of Hyperladder I (Bioline) or 2-log ladder (New England Biolabs) was run each time in the first lane of the agarose gel to estimate the size (bp) and concentration (ng/band) of the nucleic acids. The gels were then analyzed under UV light exposure using the Universal hood II gel Imager (Biorad), and the pictures were taken using the Quantity One 4.6.6 programme (BioRad).



Hyperladder I ladder



2-log ladder

Figure 10 Image of Hyper ladder I and 2-log ladder that were used during gel electrophoresis.

2.2.7 Gel Purification

In order to isolate the desired fragment of intact DNA (PCR product) from agarose gel after electrophoresis, bands were excised from the gel and purification was carried out using GeneJET PCR Purification Kit (Thermo Scientific) according to manufacturer's instructions with products eluted in 20 µL water.

2.2.8 Cloning of PCR product

In order to store the PCR products for further use, cloning was performed by using CloneJET PCR Cloning Kit (Thermo Scientific). The purified PCR product was cloned into the blunt cloning vector pJET1.2 by following the manufacturer's instructions and recovered in *E. coli* by selecting ampicillin resistant colonies.

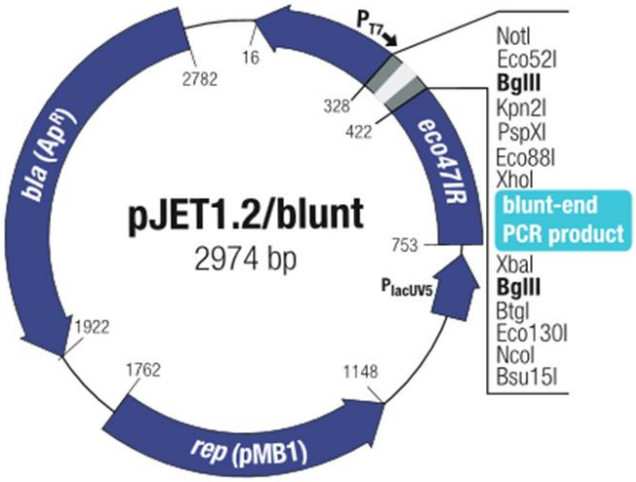


Figure 11 Map of pJET1.2/blunt.

2.2.9 Sequencing

In order to determine the nucleic acid sequences of the constructed plasmids, the plasmid samples were sent to GATC Biotech for sequencing. According to the company's instruction, the sample mixture was prepared by adding 100 ng/µL of plasmid DNA with 5 µL of primer (5 µM). The Clone Manager Suite 7 software was used to analyse the output data.

2.2.10 Protoplast-mediated transformation of *A. oryzae*

The protoplast-mediated transformation technique was carried out in order to transfer plasmids into *A. oryzae*. The *A. oryzae* strain NSAR1 (nitrate non-utilisation, sulphate non-utilisation, arginine auxotrophy and adenine auxotrophy) was used as a recipient (Jin et al., 2004). Protoplast preparation and subsequent transformation was carried out following the procedure described by (Halo et al., 2008), which employs polyethylene glycol (PEG) and calcium chloride; the appropriate auxotrophy of *A. oryzae* strain was used as a selectable marker for the transformations on CZD plates (35 g/L Czapek-dox broth, 1 M sorbitol, 1.5 g/L methionine, 0.1 g/L adenine, 2 g/L arginine, 2 g/L ammonium sulphate, 15 g/L agar; the nutrient supplement will be left out according to auxotrophy selection of each strain).

The spores of *A. oryzae* NSAR1 or *A. oryzae* transformant strains were collected from a 10-day-old plate, filtered through Miracloth and used to inoculate into 50 mL GN liquid medium (10 g/L D-glucose, 20 g/L Nutrient broth no.2 (Lab M), incubated overnight at 28^{o}C, 200 rpm. The culture was harvested by centrifugation at 8000 x g for 10 minutes. After discarding the supernatant, the pellets were resuspended in 10 mL protoplasting solution (20 g/L lysing enzyme from *Trichoderma harzianum*, 10 g/L Driselase from Basidiomycetes sp., 0.8 M NaCl), and incubated on a gently mixing roller at room temperature for 1.5 hours. The protoplasts were separated from hyphae by filtering through Miracloth. The protoplast filtrate was then centrifuged at 3000 x g for 5 minutes and resuspended in 100 µL of solution 1 (0.8 M NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl [pH 7.5]). The concentration of protoplast suspension was measured by using a haemocytometer and diluting the suspension to $1x10^7$ m/L with solution 1. A 100 µL aliquot of protoplast suspension was used for each transformation reaction. Then each transformation mixture was added with 5-10 µg of plasmid DNA and incubated on ice for 2 minutes. Afterwards, 1 mL of solution 2 (600 g/L PEG 3350, 10 mM $CaCl_2$, 50 mM Tris-HCl [pH 7.5]) was added to the mixture and incubated at room temperature for another 20 minutes.

Lastly, the appropriate melted selective CZD top medium was added and mixed with the transformation mixture prior overlay over selective CZD base medium lacking a supplement for auxotrophic selection marker. The plates were incubated at 28^{o}C for 3-5 days until the transformant colonies formed. The subculturing process of transformant colonies were performed for at least three times to ensure the stability of genes inserted of the transformant strains.

2.2.11 Transformation of *E. coli*

2.2.11.1 Preparation of electrocompetent *E. coli*

Electrocompetent cells of *E. coli* strain ccdBS were prepared prior to transformation according to the procedure published by (Nishimura et al., 1990). A colony from fresh streaked *E. coli* was inoculated into 5 mL of LB media and incubated overnight at 37°C on a rotary shaker at 225 rpm. On the next day, four 250 mL flasks each containing 100 mL of LB medium were inoculated with 1 mL overnight culture *E. coli* per flask, then incubated as before until OD_{600} = 0.3 was reached. After that, cell cultures were decanted into 8 of 50 mL Falcon tubes and centrifuged at 2500 x g for 10 min at 4°C. All manipulations were carried out with the cell on ice. After discarding the supernatants, each cell pellet was resuspended in 50 mL ice cold sterile water and centrifuged as before. After discarding the supernatants, each cell pellet was resuspended in 25 mL ice cold sterile water and the samples were pooled into 4 tubes, then centrifuged as before. After discarding the supernatants, each cell pellet was resuspended in 10 mL ice cold 20% glycerol (filter sterilized) and the samples were pooled into a single tube. After that, 10 mL ice cold 20% glycerol was added, and the tube was then centrifuged at 2000 x g for 10 min at 4°C. Finally, the cell pellet was resuspended in 1.2 mL ice cold 20% glycerol and divided into 50 µL aliquots, which were rapidly transferred into sterile Eppendorf tubes and frozen in liquid nitrogen. The cells were stored at - 80°C.

2.2.11.2 Transformation of electrocompetent *E. coli* with plasmid DNA by electroporation

Electrocompetent *E. coli* ccdBS cells were thawed on ice. Fifty µL of cells were mixed gently with 10 µL plasmid solution. The cell mixture was then added into the electroporation cuvette, wiping off excess moisture outside the cuvette and placed into the Biorad electroporator. The electroporator was set at 2500 V using the default EC2 program. After pulsing the cuvette was removed and 200 µL of SOC (0.5% [w/v] yeast extract, 2% [w/v] tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose filter sterilised) was added. Afterward, the SOC-cell mixture was transferred to an Eppendorf tube and incubated at 37°C for 1 hour. Finally, 200 µL of the transformation mixture were plated onto selective LB medium supplemented with 50 µg/mL ampicillin and incubated overnight at 37°C.

2.2.12 Yeast Homologous Recombination for plasmid construction

Yeast recombination is a technique used to recombine DNA fragments into plasmids using the yeast *S. cerevisiae*. The DNA fragments to be inserted into the backbone plasmid were amplified through PCR by using Phusion High-fidelity DNA polymerase. Primers used contained 30 bp of sequence homology to the plasmid. Backbone plasmid were extracted from *E. coli* and digested with the appropriate restriction enzyme. Yeast transformation was described by (Gietz and Woods, 2002). A single colony of yeast *S. cerevisiae* Y10000, YPH499 or AM109 was grown overnight in 10 mL YPAD liquid medium at 28^{o}C and shaken at 200 rpm. The starter culture was then used to inoculate a flask containing 40 mL of YPAD and was shaken further for another 5 hours. After that the culture was centrifuged at 8000 x g for 5 minutes. At room temperature, the pellets were washed several times with 25 mL sterile water and centrifuged as before. The water was discarded, and pellets were resuspended with 1 mL of 0.1 M LiOAc solution and transferred to 1.5 mL Eppendorf tube. Cells were pelleted again and resuspended in 400 µL of 0.1 M LiOAc solution. An aliquot of 50 µL of pellets suspension for each transformation was divided into new 1.5 mL tubes and then centrifuged.

Each transformation tube contains; 240 µL of 50% PEG 3350 solution, 36 µL of 1 M LiOAc solution, 50 µL of salmon sperm single-stranded DNA (2 mg m/L) and 34 µL of DNA fragments (typically 10 µL of linearised backbone plasmid and 5 µL of each PCR product the sterile distilled water was used to made up final volume). The mixture was mixed well and incubated at 30^{o}C for 30 minutes, then at 42^{o}C for another 30 minutes. The cell mixture was then centrifuged and resuspended in 500 µL of sterile water. The yeast transformation mixture was plated on synthetic medium SM lacking an appropriate auxotrophic marker (1.7 g/L Yeast nitrogen base without amino acid, 5 g/L ammonium sulphate, 20 g/L D-glucose, 0.77 g/L Yeast Synthetic Drop-out medium supplements, 20 g/L agar) and incubated at 28^{o}C for 2-3 days. The transformant yeast colonies were collected and yeast plasmid DNA purification was carried out using the Zymoprep Yeast Plasmid Miniprep I as described previously. Purified yeast plasmid was rescued into *E. coli* competent cells in order to have a higher number of copies. After *E. coli* transformation, colony PCR was carried out to check for the presence of the desired DNA from the obtained transformation colonies. The positive colonies were then purified, and restriction digestion reaction was performed to confirm the correct construction.

2.2.13 Plate-based assay for assessing antibacterial activity

The plate-based assay against *Bacillus subtilis* was used to study an antibacterial activity of the fungal transformant strains following the procedure as defined by (Hartley et al., 2009). In brief, spores of *B. subtilis* were prepared by inoculating 50 mL TSB broth and grown overnight at 28°C. The horizontal pre-prepared TSA agar base medium in a Thompson bottle was inoculated over the surface with one mL of the overnight culture and incubated at 30°C for 6 days. The spores of *B. subtilis* were harvested using 100 mL of sterile distilled water. The spores were then activated by heat at 70°C for 30 minutes and stored at 4°C until needed.

To assess an antibacterial activity of the fungal transformants strains, A plug of each transformant strain was used to inoculate onto the central of CMP agar plates (35 g/L Czapek-dox broth premix, 20 g/L maltose, 10 g/L peptone, 15 g/L agar) and grown at 28°C for 3 days. Five mL of melted TSAg (30 g/L tryptic soy broth premix, 5 g/L agar, 10 g/L glucose) overlay medium containing 5 µL of B. subtilis spores and 37.5 µL of 4 % TTC (2,3,5-Triphenyl-2H-tetrazolium chloride) was poured onto each plate and allowed it to solidify to form uniform layer. The plates were then incubated overnight at 28°C, and the clearing zone were observed and measured.

2.2.14 Golden Gate Assembly protocol

Golden Gate reactions were set up for generating the final expression vector containing genes involved in the Pleuromutilin biosynthetic pathway as described by (Weber et al., 2011). Restriction-ligation reactions were set up in one tube reactions containing: ~100 ng of acceptor plasmid, ~200 ng of inserted PCR product/ or each DNA component of plasmid containing each module (2:1 molar ratio was the appropriate ratio for insert and acceptor vector), 1 µL of required restriction enzyme (*Bsa*I or *Bpi*I), 1 µL of T4 DNA ligase enzyme, 2 µL T4 DNA ligase buffer, 2 µL 10X FastDigest Green Buffer and made up to final volume of 20 µL with sterile water.

The reaction was performed in a thermocycler using the following program: initial incubation at 37^{o}C for 30 seconds, then incubated at 37^{o}C for 3 minutes and 16^{o}C for 5 minutes, both steps repeated 50 times, followed by incubation at 50^{o}C for 5 minutes and 80^{o}C for 5 minutes. The reaction mixture was transformed into competent *E. coli* cells and the transformation was plated on LB plates containing the appropriate antibiotic. For blue/white

selection, plates were supplemented with X-gal (2% w/v dissolved in DMSO) prior to plating.

2.2.15 *Agrobacterium* infiltration of *N. tabacum*

The *Agrobacterium* infiltration was performed as described in Voinnet et al., (2003). *Agrobacterium tumefaciens* LBA4404 wild type strain and transformant strains containing the pL2 expression vector were streaked on LB agar plates containing appropriate antibiotic and grown at 28°C for 48 hours. A single colony was picked and inoculated into 5 mL of LB media supplemented with appropriate antibiotic and incubated further at 28°C for 48 hours with 300 rpm. After that 25 mL of LB media plus 10 mM MES pH 5.7 (adjusted with KOH), 150 µM acetosyringone and the selective antibiotics was inoculated with 1 mL overnight culture and grown overnight at 28°C. Subsequently, the bacterial pellets were precipitated and washed once with the equal volume of MES buffer pH 5.7 and then resuspended in MMA buffer (10 mM $MgCl_2$, 10 mM MES pH 5.7, 150 µM acetosyringone). The OD_{600} was adjusted to 0.5-1.5 and the bacterial cells were stored at room temperature for 2-5 hours prior used for infiltration.

In order to perform an *Agrobacterium* syringe infiltration, the 4-6 -week-old *N. tabacum* plants were selected for transformation. Leaves were gently infiltrated with *A. tumefaciens* using a syringe to force the solution through the stomata of the lower leaf surface. A successful infiltrate was observed as a spreading wetting area in the leaves. The infiltrated plats were then maintained in a growth chamber at 25ºC with a 16 h/ 8 h of light/dark photoperiod condition for 3 days. Afterwards, the infiltrated region on leaves were harvested for further analysis.

2.3 Chemical analysis

2.3.1 Fungal chemical extractions

Chemical extraction of fungal culture was performed in order to assess the production of metabolites by fungal strains. A 100 mL of CMP broth in a 250 mL flask was inoculated with 1 mL of $2.5x10^7$ m/L spore solutions from NSAR1 and from *A. oryzae* transformant strains, and grown on shaking incubator at 28^{o}C, 200 rpm for ten days. Growing mycelia cultures of *A. oryzae* NSAR1 and *A. oryzae* transformants were each harvested and homogenised after adding an equal volume of water. Then concentrated HCl was used to adjust the pH to a value of 3 units. Following that an equal volume of ethyl acetate was added to the culture, and the mixture was stirred for 20-30 minutes at room temperature. The organic phase and water phase were separated in a separating funnel. While the organic phase was collected, the water phase was then extracted again with an equal volume of ethyl acetate for three times and these organic extracts were pooled.

The organic extract was dried using anhydrous $MgSO_4$ and concentrated by evaporating using a rotary evaporator at 30^{o}C, to remove the solvent. The dried crude extract was resuspended in methanol (10 mg m/L), and an equal volume of hexane was added, followed by shaking in a separation funnel to allow the defatting of the sample. The methanol phase was subjected to two additional rounds of defatting, while the hexane phase was removed. A pre-weighted vial was used to collect the methanol phase and dried under a flow of dry N_2 gas and weighted again. Dried crude extract was resuspended in acetonitrile to a concentration of 10 mg m/L and transferred to a LC-MS vial.

2.3.2 Yeast chemical extractions

Chemical extractions with hexane were undertaken on yeast liquid cultures in order to analyse the metabolite produces by the yeast transformant strains. The extraction procedure was applied as described by (Zhuang and Chappell, 2015). The yeast culture was added with the equal volume of acetone and mixed vigorously for 5 minutes to lyse the cells. The cell mixture was then incubated at room temperature for 10 minutes. Following that, equal volume of hexane was added and mixed well again, the mixture was then centrifuged for 5

minutes at maximum speed. The organic layer was collected and concentrated under a N_2 stream before being analysed through TLC.

2.3.3 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was performed to optimised an appropriate solvent system, used to separate the components of a compound from crude extract mixture. It was also used as an alternative method to screen transformant prior to LC-MS and for GC transformants where LC-MS failed to detect the product. The crude extract was spotted onto a 2x10 cm aluminium-backing TLC plate (TLC silica gel 60 F254; Merck, Darmstadt, Germany), and developed in an appropriate ratio of petroleum ether and ethyl acetate. To visualise the compounds and recorded the *Rf*, the plate was immersed in potassium permanganate solution (1.5 g of $KMnO_4$, 10 g of K_2CO_3 and 1.25 mL of 10% NaOH in 200 mL of water).

2.3.4 Liquid chromatography-mass spectrometry (LC-MS) analysis

The LC-MS was performed to screen the crude extracts from fungal transformants for the production of metabolite compounds. The analysis was performed on a Waters 2767 HPLC system with a Waters 2545 pump system. Chromatographic separation was achieved using a Phenomenex LUNA column (2.6 µ, C18, 100 Å, 4.6 × 100 mm) and a Phenomenex Security Guard precolumn (Luna C5 300 Å) for reverse-phase chromatography. UV absorbance was detected between 200 – 400 nm using the Waters 2998 diode array detector. Mass spectrometry data was calculated using the Waters Quattro Micro spectrometer, for detecting an m/z (mass/charge) range between 150 and 800 Da in positive (ES+) and negative (ES-) mode.

The gradient elution conditions were; A, HPLC grade H_2O containing 0.05% formic acid; B, HPLC grade CH_3CN containing 0.045% formic acid) with the following program: 0 minutes, 20% B; 15 minutes, 90% B; 16 minutes 95% B; 17 minutes 95% B; 18 minutes 10% B, 20 minutes 10% B. Flow rate was set at 1 mL min-1. The data were acquired using Waters MassLynx™ V4.1 Software.

2.3.5 Preparative High-performance liquid chromatography (HPLC) analysis

The preparative HPLC was carried out to detect a specific metabolite from a fungal crude extract. The HPLC system was the same one used in the LC-MS analysis, but the separation and quantitation have been done on a Phenomenex LUNA column (5µ, C18, 100 Å, 10 × 250 mm). The mobile phase was prepared by mixing a gradient of solvents (A, HPLC grade H_2O containing 0.05% formic acid; B, HPLC grade CH_3CN containing 0.045% formic acid). The elution program was performed with 0 minutes, 5% B; 2 minutes, 10% B; 20 minutes, 90% B; 21 minutes 95% B; 26 minutes 95% B; 27 minutes 5% B, 30 minutes 5% B, with flow rate at 16 mL/min. The injection volume was 20 µL. The UV absorbance was set at 200-400 nm and detected with a Waters 2998 diode array detector, while mass range from 100-600 Da in positive (ES+) and negative (ES-) mode was detected by a Waters Quattro Micro spectrometer, and approximate amount of the eluent was estimated with Waters 2424 for ELSD.

2.4 Bioinformatics

The sequence data of all the genes worked in this project were visualised through Benchling software. Primers design and selection of enzymes for the restriction digestion reaction were also done by this program. The program also allows to plan and design the expression vector for the Golden Gate cloning and the cloning reaction to build the recombinant plasmids. All plasmid maps of the expression vectors were drawn using GENtle V1.9.4 software.

Chapter 3 New promoters for improving pleuromutilin production in *Aspergillus oryzae*

3.1 Introduction

3.1.1 Biosynthetic evolution of pleuromutilin / production

Ongoing problems with antibiotic resistance in the 1980s led to numerous drug derivatives being licensed from the existing antibiotic classes (Alanis, 2005). However, the issue of cross-resistance between the drugs of the same class continued to increase (Novak, 2011). The worst example came from the third generation of cephalosporins, where resistance was found in 1983, showing that SHV-2 enzyme was capable of hydrolysing these drug classes and this resistance spread rapidly (Kliebe et al., 1985, Knothe et al., 1983, Philippon et al., 1989). Pleuromutilins have regained attention as a class of antibiotics following recent extensive biological and chemical characterisation. Many attempts have been made to improve and develop their antibacterial activity and the first pleuromutilin antibiotic for human use, retapamulin, made its way onto the market for topical use in humans for the treatment of skin infections like impetigo in 2007 (Jacobs, 2008).

Pleuromutilin and its derivatives are known for an antibacterial activity against gram-positive cocci bacterial, such as *Staphylococcus aureus*, *Streptococcus haemolyticus*, and *Bacillus subtilis* (Kilaru et al., 2009). Further study also showed that the compounds were not only active against gram-positives but also active against gram-negative species such as *Mycoplasma spp* (Novak, 2011). Pleuromutilin has a unique mode of action via an interaction with the 50S ribosomal subunit, which inhibits prokaryotic protein synthesis. This make the drug attractive for further development as an antibiotic for human use (Shang et al., 2014).

The approval process for pleuromutilin was very slow because it faced numerous problems around its metabolic instability, cardiac and hepatotoxicity concerns and insufficient oral bioavailability. Together these issues made it very challenging for product development and can explain why it had a poor perception in the pharmaceutical industry and resulted in a very slow progress in its development (Novak, 2011).

In the last two decades, successful efforts in the development of semi-synthetic pleuromutilin drugs for human use were made by GSK (retapamulin, Figure 12 A-B) and Nabriva

Therapeutics (BC-3781 or lefamulin, Figure 12 C-D) (Prince et al., 2013, Rittenhouse et al., 2006). These semisynthetic compounds were established through the extensive focus on SAR studies, along with the previous reports that pointed out that the nature of the C14 side chain could be an important key to determine its systemic efficiency (Egger and Reinshagen, 1976). Other reports have illustrated the correlation between C14 side chain and its impact on absorption, distribution, metabolism, excretion and toxicity (ADMET) parameters (Novak and Shlaes, 2010). In late 2006 lefamulin (Figure 12C), a thioether compound was first synthesized by Nabriva Therapeutics in two dosage forms: oral and intravenous (Figure 12D). In 2011, it was the first systemic pleuromutilin to be tested successfully in clinical phase II trials. In August 2019, lefamulin (Xenleta^{TM}) was approved by FDA to treat Community-acquired bacterial pneumonia (CABP) in adult. It is the first systemic pleuromutilin antibiotic to have been launched into the market for IV and tablet use. (Press Statement by Nabriva Therapeutics, 2019).

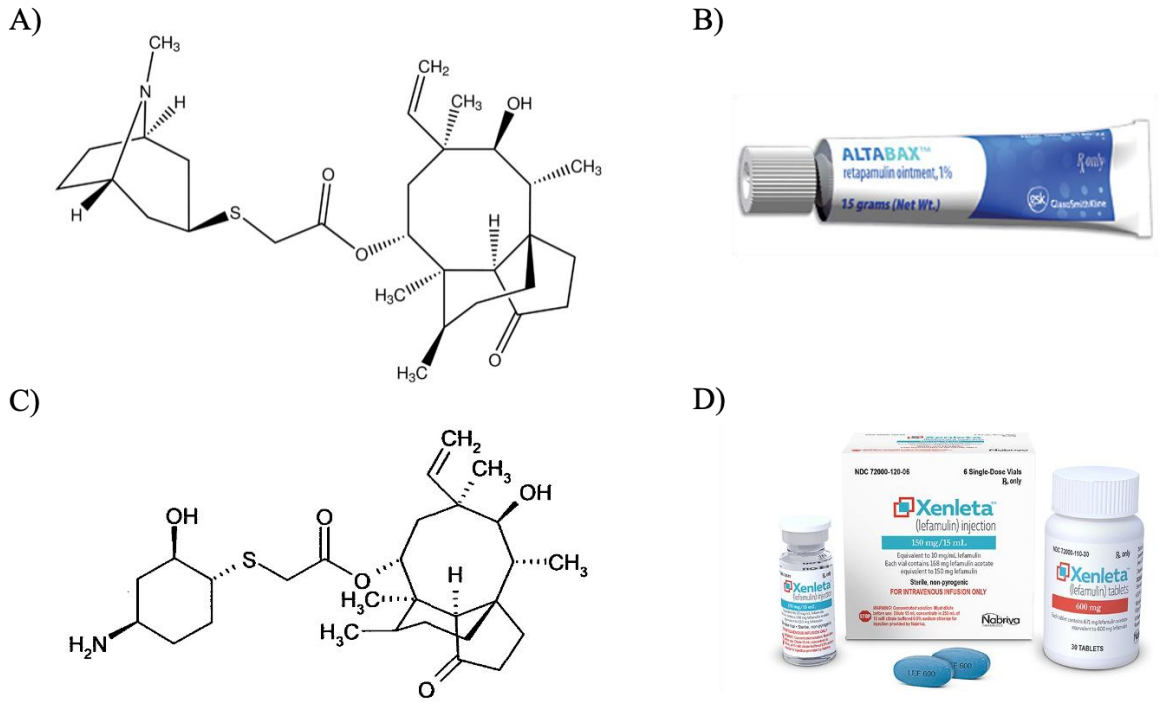


Figure 12 A) Chemical structure of retapamulin B) Topical ointment form of retapamulin launched in the market C) Chemical structure of lefamulin D) Tablets and injection dosage forms of lefamulin.

Since lefamulin is recently approved and launched into the market in both oral and intravenous dosages, this might lead to an increasing demand for the antibiotic in the future. One constraint on its use is that the commercial cost is high due to its low yield and semisynthetic modifications. The company announced that the injectable version would cost $205 per day, while the oral version would be $275 per day (Saumya, 2019). Pleuromutilin is a tricyclic compound that is naturally produced by *C. passeckerianus*, but due to its difficulty to grow under normal laboratory conditions, it usually requires a long period of fermentation and produces a very low titre (Nagamine et al., 2019). Therefore, seeking a strategy to increase the titre of pleuromutilin production for mass fermentation is urgently needed.

The genetic techniques for transformation and gene manipulation for basidiomycetes have already been developed and reported (de Mattos-Shipley et al., 2011, Kilaru et al., 2009). Further study on characterization of genes responsible for pleuromutilin biosynthesis pathway was done by Bailey and Foster's group (Bailey et al., 2016). The λ-phage genomic DNA library of *C. passeckerianus* was screened and the expression analysis has illustrated that - GGS, Cyclase, P450-1, P450-2, P450-3, ATF and SDR were the seven genes responsible for pleuromutilin synthesis. The result was proven by the heterologous expression of the seven genes in the secondary host, *A. oryzae* giving production of pleuromutilin. Surprisingly, *A. oryzae* transformant strains containing the seven genes of pleuromutilin synthesis showed a significant increase of yield for pleuromutilin production compared to the natural host *C. passeckerianus*.

Alberti et al., (2017), showed that silencing of the genes in *C. passeckerianus* to determine the function of each gene in the pathway was not a suitable method. Once a single gene in the cluster was silenced, it triggered the downregulation of the other genes involved in the pleuromutilin pathway and led to no accumulation of any intermediates that could be detected. In order to investigate the role of each gene, the heterologous expression of genes in *A. oryzae* and chemical analysis for an intermediate product were employed. Based on these results, levels of GGS and cyclase were the likely bottleneck for the pleuromutilin biosynthetic pathway. The expression of these two genes would control the entry of the main precursor into the pathway.

Geranyl-geranyl diphosphate synthase (GGS) was demonstrated to be responsible for production of the precursor geranyl geranyl diphosphate (GGPP). Following that, cyclase (CYC) would catalyse the cyclization of GGPP. The expression of these two genes resulted

in the production of the first pathway intermediate, 3-deoxo-11-dehydroxy-mutilin (Figure 13). This compound was the first committed step in the pleuromutilin biosynthesis pathway. The cytochrome P450s were proved to be involved in the pleuromutilin pathway. Their key role was to introduce modifications into the core structure of pleuromutilin. P450 enzymes are well known as a large group of enzymes that are commonly found in secondary metabolite biosynthesis pathway of bacteria, fungi and plants. These enzymes generate the complexity in the core structure by various reactions for example; oxidation, dehydration, epoxidation, C-C cleavage and etc (Hu et al., 2011, Lin et al., 2014, Seki et al., 2008). In the pleuromutilin biosynthesis cluster, there are three P450 genes: *P450-1*, *P450-2* and *P450-3*, which were proved to be involved in the pathway. The P450-1 and P450-2 acted first by introducing the hydroxyl groups on C11 and C3 of the scaffold respectively (Figure 13).

During the later stages of the pleuromutilin pathway, the short-chain dehydrogenase/reductase (SDR) was discovered to catalyse and convert the hydroxyl group at C3 into a ketone group. Subsequently, acetyl transferase (ATF) was determined to be a part of the pathway, adding the acetyl group to the mutilin core at C14 to yield the last intermediate of the pathway, 14-O-acetyl mutilin. The final step was done by the action of P450-3 with a hydroxylation reaction to introduce a hydroxyl group on C22, converting the 14-O-acetyl mutilin intermediate to the complete structure of pleuromutilin (Alberti et al., 2017). Even though the heterologous expression in *A. oryzae* strains harbouring the seven genes of pleuromutilin biosynthesis pathway was successful, the total yield was still lower than expected and not suitable for mass production.

In addition, the Alberti et al. (2017) study also performed a feeding experiment to explore the hypothesis that feeding the engineered *A. oryzae* stains with synthetic intermediates would provide the opportunity to gain a high efficiency of semisynthetic precursor conversion into other new intermediates. This could be a chance to reduce the number of steps involved in the semisynthetic process that is currently employed in pleuromutilin production. The results demonstrated that this was successful, but the yield was still very limited (Alberti et al., 2017).

The Alberti et al., (2017) study also indicated that the poor yield might due to the use of the enolase promoter to drive the expression of genes Cyclase, P450-3 and SDR within the pathway. This promoter showed the lowest expression level out of the three promoters (*Padh*, *Pgpd* and *Peno*) and thus may be a rate limiting step. In other words, the limitation of the

power of enolase promoter could lead to insufficient levels of Cyclase, P450-3 and SDR, thereby constraining yield of the final product. Therefore, an attempt to increase the titre of pleuromutilin by enhancing the promoter expression level is a priority.

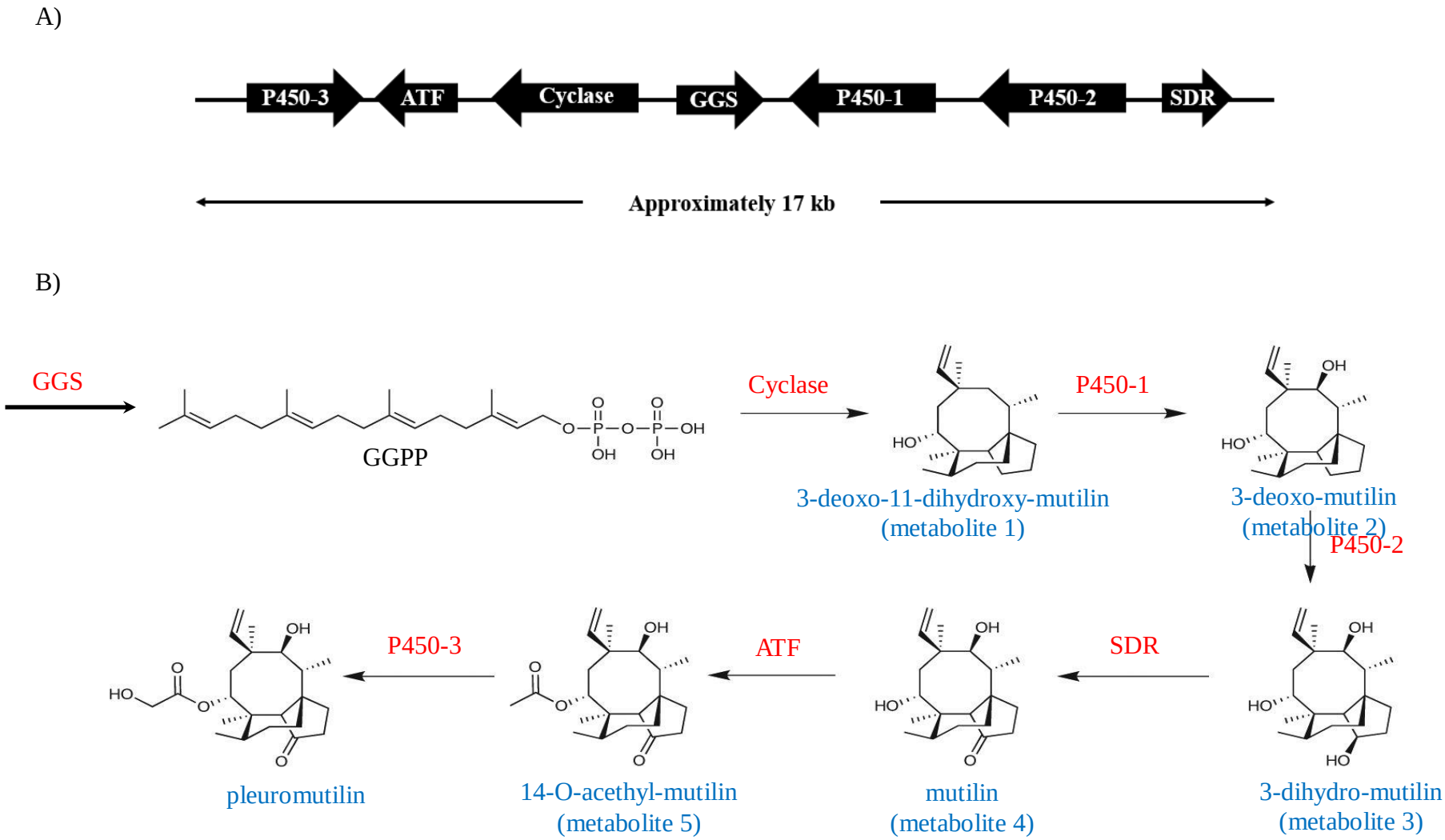


Figure 13 A) The seven genes involved in the pleuromutilin biosynthesis pathway B) Proposed biosynthetic pathway of pleuromutilin showing the gene for each step and each intermediate that exists along the pathway. Adapted from (Alberti et al., 2017).

3.2 Aims

The aim of this chapter was to enhance the production of pleuromutilin in the secondary fungal host *Aspergillus oryzae*. Feeding experiments with synthetic intermediates were also performed with semisynthetic intermediates to find new compounds that have better antibiotic activity.

In order to complete the aim, several objectives were set as listed below:

1. To recreate the expression vectors by replacing the enolase promoter activity involved in the efficient expression of the genes responsible for the biosynthesis of pleuromutilin in the secondary host *A. oryzae*
2. *A. oryzae* transformation for the heterologous expression of the new expression vectors
3. Preliminary bioassay screening against *Bacillus subtilis* for antibiotic activity of *A. oryzae* transformant strains
4. Feeding experiments using *A. oryzae* transformant strains with a semisynthetic intermediate, to assess the capability of gene under control of the *pdc* promoter in order to convert the substrate to a desired compound
5. Chemical analysis to evaluate yield improvement in the production of the metabolite extracted from *A. oryzae* transformant strains under the control of new promoter compared with the previous strains under the control of *Peno* promotor

3.3 Results

3.3.1 Promoter replacement to alter heterologous gene expression in *A. oryzae*

The previous studies within our group showed fairly low titres of pleuromutilin production from *A. oryzae*, and the results indicated that weak expression from genes under the control of the enolase promoter might be rate-limiting. Therefore, my study was to identify better promotors to see if higher titres could be obtained by enhancing the expression of these genes.

For efficient expression in *A. oryzae*, it is essential that the expression of the target gene be driven by an active promoter either derived from the host or a related species. Various promoters of *A. oryzae* have been reported for efficient recombinant protein production (Nevalainen and Peterson, 2014).

The expressed sequence tag (EST) database analysis of *A. oryzae* (Akao et al., 2007, Wang et al., 2010) was used as a source for the candidate promoters of *A. oryzae* for my project, selecting the most abundantly expressed genes under liquid growth. Promotors chosen were; *ptrA* gene for thiamine thiazole synthase, an enzyme that is involved in thiamine biosynthesis, *pgkA* gene for phosphoglycerate kinase, involved in the glycolysis pathway which synthesizes pyruvate from D-glyceraldehyde 3-phosphate, and *pdcA* gene for pyruvate decarboxylase, an enzyme that converts pyruvate into acetaldehyde and carbon dioxide. These promoters were predicted to have strong activity in *A. oryzae* for the expression of foreign genes in liquid culture. Before using these promoters with the desired pathway genes, the expression of each promoter was investigated for its ability to drive expression of the reporter gene GFP through the method shown in Figure 14, to assess GFP expression under different conditions.

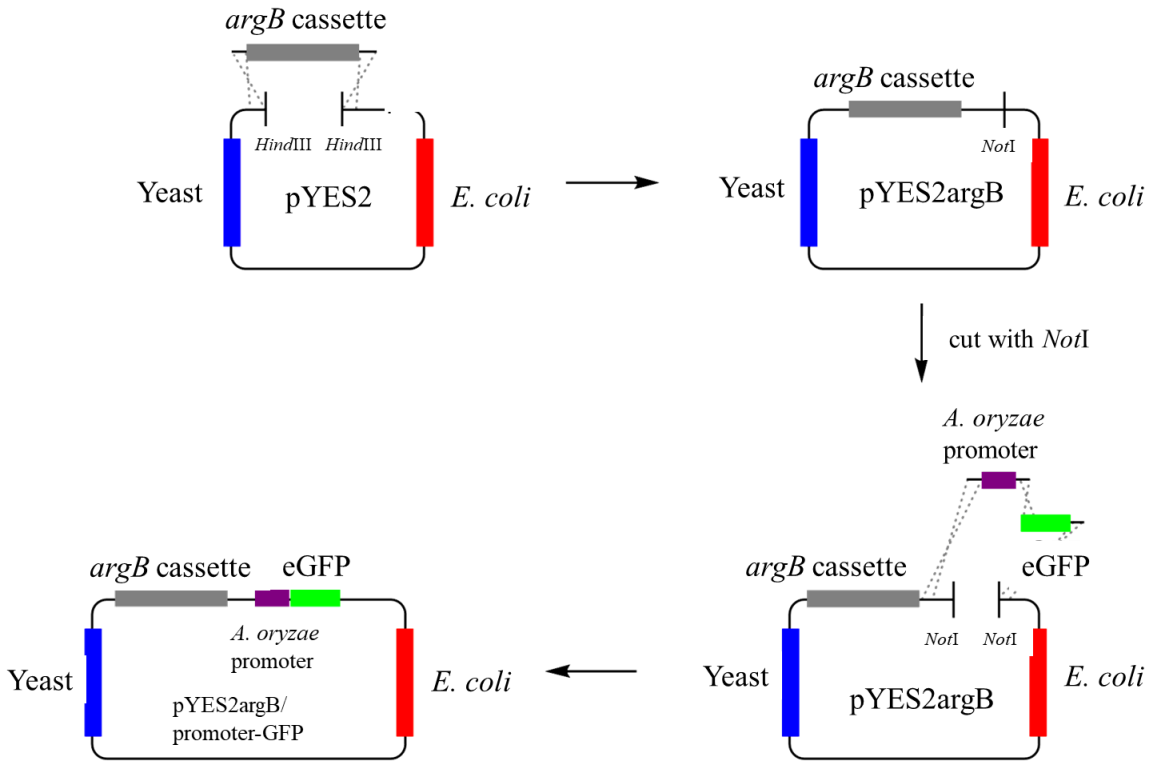


Figure 14 Principle of promoter expression cassettes. Promoter expression cassettes consist of a promoter of choice (*ptr*, *pgk* and *pdc*), reporter gene (GFP) and a selectable transformation marker (*argB* as auxotrophic host).

First, yeast homologous recombination was performed in order to build this plasmid and insert *argB* into the backbone pYES2. The *argB* genes was amplified through PCR reaction with regions of 30 bases homology to the target backbone, by using specific primers. Yeast homologous recombination was performed to join the gene with the backbone plasmid, which had been previously digested with the restriction enzyme *Hind*III. Afterwards, the pYES2argB backbone was cut with the restriction enzyme *Not*I. Then another yeast homologous recombination was carried out in order to recombine the promoter and GFP gene together along with the backbone plasmid.

3.3.2 Adapting pYES2 plasmid for *A. oryzae* transformation

The pYES2 plasmid was first adapted with *argB* so it could be used in *A. oryzae*, then the selected promotor and GFP were recombined in (see principle in Figure 14).

Yeast homologous recombination was carried out in order to build the plasmid pYES2argB (Figure 15A) by inserting *argB* gene into the backbone pYES2. The *argB* gene was amplified through PCR reactions, by using the primer pair ArgB FF*/ArgB RR* and the plasmid pTYGSarg was utilised as a template. Yeast homologous recombination was performed to merge the gene together along with the backbone plasmid, which had been previously digested with the restriction enzyme *Hind*III. All yeast colonies from the transformation plate were pooled, plasmid DNA extracted and transformed into *E. coli*. The plasmids obtained from *E. coli* were checked by PCR for the presence of the *argB* genes, with the same primers used to amplify the genes for yeast homologous recombination. The expected band of 1.5 kb was amplified from each sample. Correct construction of the plasmid was also confirmed with restriction digest with the appropriate enzyme (see Figure 15B).

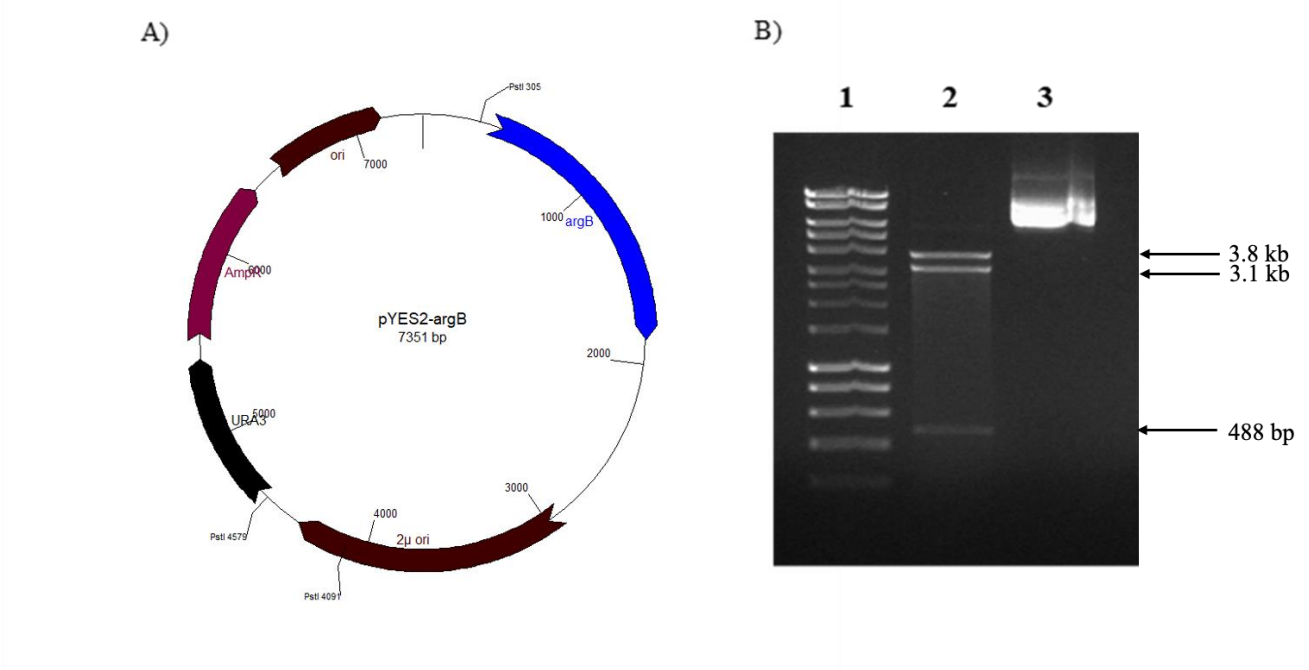


Figure 15 A) Plasmid map of the expression vector pYES2argB. This plasmid was built starting from the backbone pYES2. B) Restriction digest showing correct construction of plasmid pYES2argB. Lane 1 contains 5 µL of hyperladder I (Bioline). Lane 2 contains 10 µL of digestion reaction with restriction enzyme *Pst*I of pYES2argB (expected fragments 3.8 kb, 3.1 kb and 488 bp). Lane 3 contains 1 µL of undigested plasmid DNA of pYES2argB.

To confirm that the pYES2argB vector was functional, *A. oryzae* was transformed with the plasmid pYES2argB, which contains the *argB* gene as a selectable marker. At least five transformant colonies were obtained and subcultured onto selective medium for two consecutive times. The gDNA of each transformant was extracted with the rapid DNA extraction method. A PCR was performed using specific pairs ArgB FF*/ArgB RR* directed towards the *argB* gene. The *argB* fragment was amplified from all five *A. oryzae* transformants containing pYES2argB. It could be clearly demonstrated that the pYES2argB vector was suitable as a backbone for the insertion of promoter-GFP genes.

3.3.3 Plasmid construction for expressing GFP under the control of various *A. oryzae* promoters

Three plasmids for the expression of GFP were constructed: 1) *ptr-GFP* carried 0.7 kb *ptr* promoter followed by 0.7 kb *GFP*, 2) *pgk-GFP* that carried 0.5 kb *pgk* promoter followed by 0.7 kb *GFP*, and 3) *pdc-GFP* carried 0.5 kb *pdc* promoter followed by 0.7 kb *GFP*.

Yeast homologous recombination was carried out in order to build this plasmid and insert promoter and GFP gene into the backbone pYES2argB. The *ptr* promoter and GFP genes were amplified through PCR reactions, by using the primer pair ptrA FF(1)*/ ptrA RR(1) and prtA/GFP FF(2)/ ptrA/GFP RR(2)*, the *pgk* promoter and GFP genes were amplified by utilising the primers pgkA FF(1)*/ pgkA RR(1) and pgkA/GFP FF(2)/ pgkA/GFP RR(2)* and the *pdc* promoter and GFP genes were amplified through primers pdcA FF(1)*/ pdcA RR(1) and pdcA/GFP FF(2) pdcA/GFP RR(2)*. High-fidelity PCR amplification was carried out using the gDNA of *A. oryzae* NSAR1 as a template. Yeast homologous recombination was performed to merge the promoter fragment and GFP fragment together along with the backbone plasmid, which had been previously digested with the restriction enzyme *Not*I. All colonies from the transformation plate were pooled, plasmid DNA extracted and transformed into *E. coli*. The plasmids obtained from *E. coli* were checked with PCR for the presence of the promoter gene.

As a result, three out of twenty-eight transformant colonies showed the successful construct of pYES2arg/ptr-GFP plasmid. Correct construction of pYES2argB/ptr-GFP plasmid (Figure 16A). was confirmed with restriction digest with the appropriate enzyme (see Figure 16B).

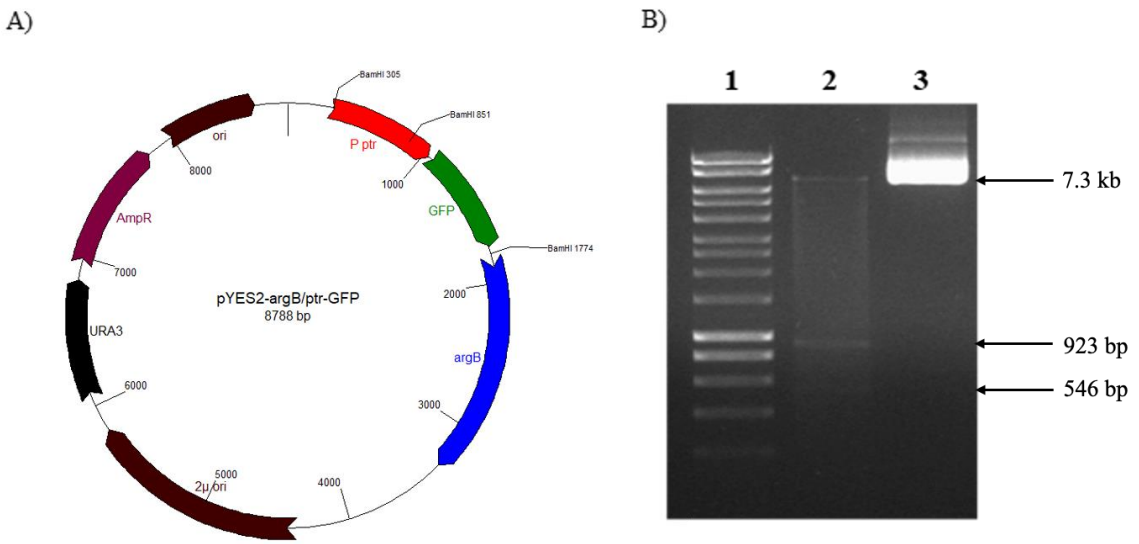


Figure 16 A) Plasmid map of the expression vector pYES2argB/ptr-GFP with GFP gene under control of *ptr* promoter. B) Restriction digest showing correct construction of plasmid pYES2argB/ptr-GFP: Lane 1 contains 5 µL of hyperladder I (Bioline). Lane 2 contains 10 µL of digestion reaction with restriction enzyme *BamH*I of pYES2argB/ptr-GFP (expected fragments 7.3 kb, 923 bp and 546 bp). Lane 3 contain 1 µL of undigested plasmid DNA of pYES2argB/ptr-GFP.

Three out of fifty-six transformants colonies showed the successful inserted *pgk* promotor and GFP gene. Correct construction of pYES2argB/pgk-GFP plasmid (Figure 17A) was confirmed with restriction digest with the appropriate enzyme (see Figure 17B).

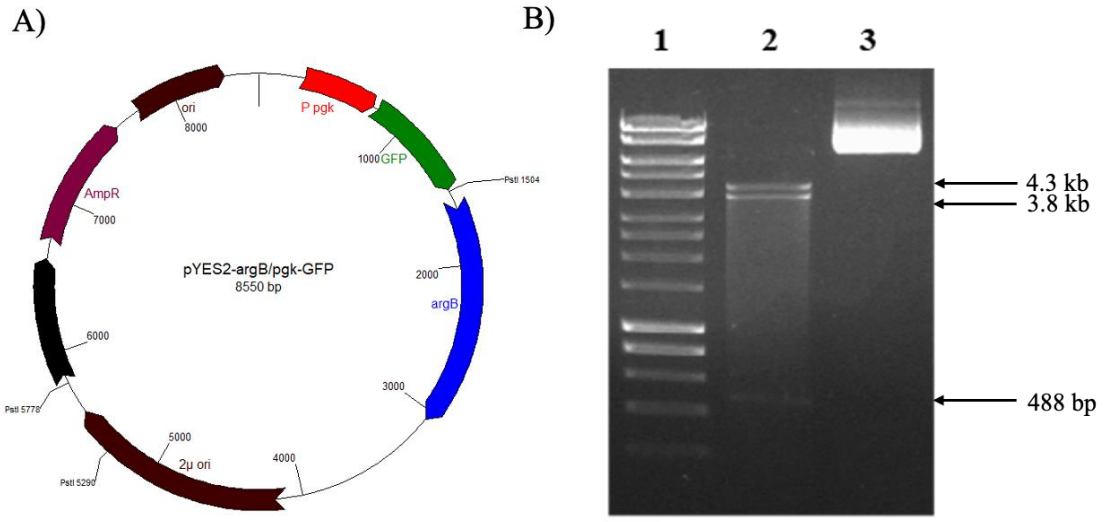


Figure 17 A) Plasmid map of the expression vector pYES2argB/pgk-GFP with GFP gene under control of *pgk* promoter. B) Restriction digest showing correct construction of plasmid pYES2argB/pgk-GFP: Lane 1 contains 5 µL of hyperladder I (Bioline). Lane 2 contains 10 µL of digestion reaction with restriction enzyme *Pst*I of pYES2argB/pgk-GFP (expected fragments 4.3 kb, 3.8 kb and 488 bp). Lane 3 contain 1 µL of undigested plasmid DNA of pYES2argB/pgk-GFP.

Two out of thirty transformant strains showed the successful construct of pYES2argB/pdc-GFP plasmid. Correct construction of pYES2argB/pdc-GFP plasmid (Figure 18A) was confirmed with restriction digest with the appropriate enzyme (see Figure 18B).

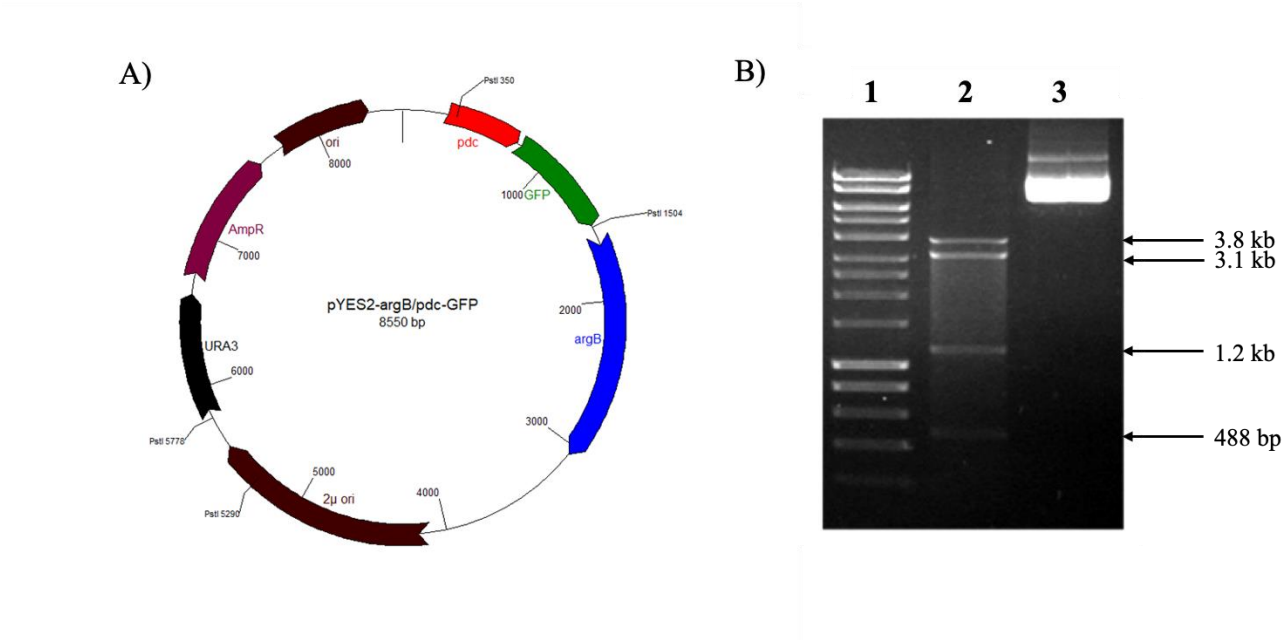


Figure 18 A) Plasmid map of the expression vector pYES2argB/pdc-GFP with GFP gene under control of *pdc* promoter. B) Restriction digest showing correct construction of plasmid pYES2argB/pdc-GFP: Lane 1 contains 5 µL of hyperladder I (Bioline). Lane 2 contains 10 µL of digestion reaction with restriction enzyme *Pst*I of pYES2argB/pdc-GFP (expected fragments 3.8 kb, 3.1 kb, 1.2 kb and 488 bp). Lane 3 contains 1 µL of undigested plasmid DNA of pYES2argB/pdc-GFP.

3.3.4 Transformation of *A. oryzae* to investigate the expression levels of GFP under the control of various promoters

The transformation of *A. oryzae* (strain NSAR1) with plasmid pYES2argB/ptr-GFP, pYES2argB/pgk-GFP and pYES2argB/pdc-GFP were made separately in order to assess expression of the GFP under the control of different promoters. The transformant colonies were obtained and subcultured onto selective medium for two consecutive times. Fluorescence of stable transformants were observed under fluorescence microscope, and the transformants containing pYES2argB with no GFP were used as a negative control (figure not shown). From five transformants of pYES2arg/ptr-GFP only one strain showed the expression of GFP in their mycelia (Figure 19A). While two out of five transformants of pYES2arg/pgk-GFP showed the GFP expression (Figure 19B). Five out of ten transformants of pYES2arg/pdc-GFP showed a strong fluorescence expression (Figure 19C). The transformant colonies containing pYES2arg/pdc-GFP showed the stronger expression of GFP in their mycelia when compared with the transformant colonies contain pYES2argB/ptr-GFP and pYES2argB/pgk-GFP. Therefore, the *pdc* promoter was chosen to use as a new promoter to replace and drive the genes that previously under the control of *eno* promoter in the pleuromutilin biosynthesis pathway.

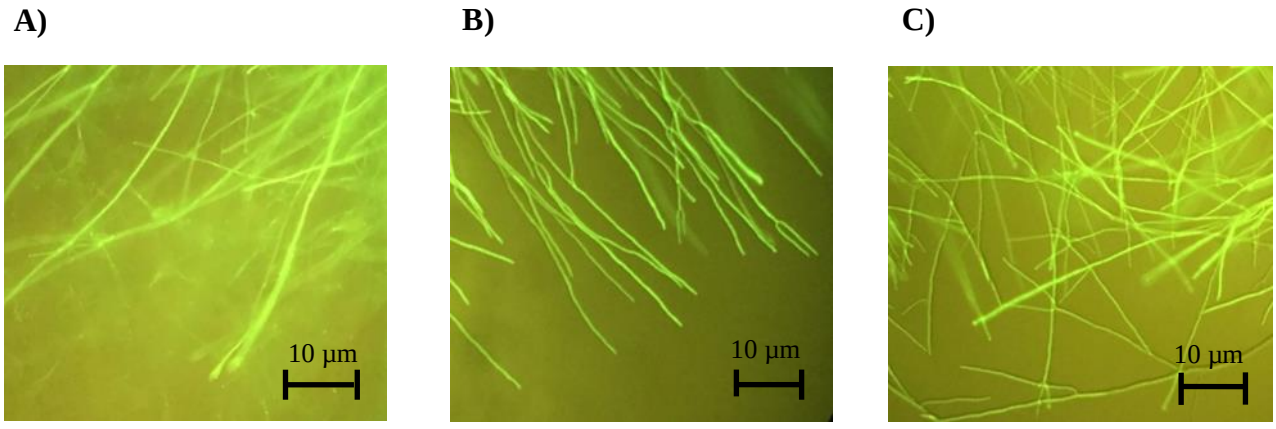


Figure 19 Fluorescence of GFP in the hyphae of representative *A. oryzae* transformants. A) pYES2argB/ptr-GFP vector. B) pYES2argB/pgk-GFP vector. C) pYES2argB/pdc-GFP vector.

3.3.5 Construction to recreate the three new plasmids under the control of pdc promoter for Cyclase, P450-3 and SDR genes

There were three genes under the control of the enolase promoter (*Peno*) in the previous expression vectors: Cyclase, P450-3 and SDR, (Table 5 indicated in red). Their expression vectors were redesigned to replace *Peno* with the *pdc* promoter. Using the yeast recombination method, pTYGSargGGSCyclase, pTYGSadeP1P2P3 and pTYGSbarATFSDR were used as backbones and were cut with *Spe*I. The *pdc* promoter region for driving expresion of Cyclase, P450-3 and SDR was amplified using primer pair; pdcA-CYC FF*/ pdcA-CYC RR*, pdcA-P450-3 FF*/ pdcA-P450-3 RR* and pdcA-SDR FF*/ pdcA-SDR RR* for each gene, respectively. The correct construction of three new vectors: pTYGSargGC-Ppdc, pTYGSadeP1P2P3-Ppdc and pTYGSbarAS-Ppdc was confirmed by PCR for the presence of the *pdc* fragment. Restriction digestions with appropriate enzymes were also done to confirm the correct assembly (Figure 20).

Table 5 Details of the previous plasmids used by Dr. Alberti to express the seven genes of the pleuromutilin pathway in *A. oryzae*. Selective markers; *argB* - Ornithine carbamoyltransferase, *adeA* -gene which encodes purine biosynthesis and *bar* - Phosphinothricin acetyl transferase gene. Promoters; *adh* -Alcohol dehydrogenase, *gpdA* - Glyceraldehyde-3-phosphate dehydrogenase and *eno* -enolase.

Plasmid	Selective marker gene	Promoter	Gene
pTYGSargGC	*argB*	Padh	GGS
		Peno	Cyclase
pTYGSadeP1P2P3	*adeA*	Padh	P450-1
		PgpdA	P450-2
		Peno	P450-3
pTYGSbarAS	*bar*	Padh	ATF
		Peno	SDR

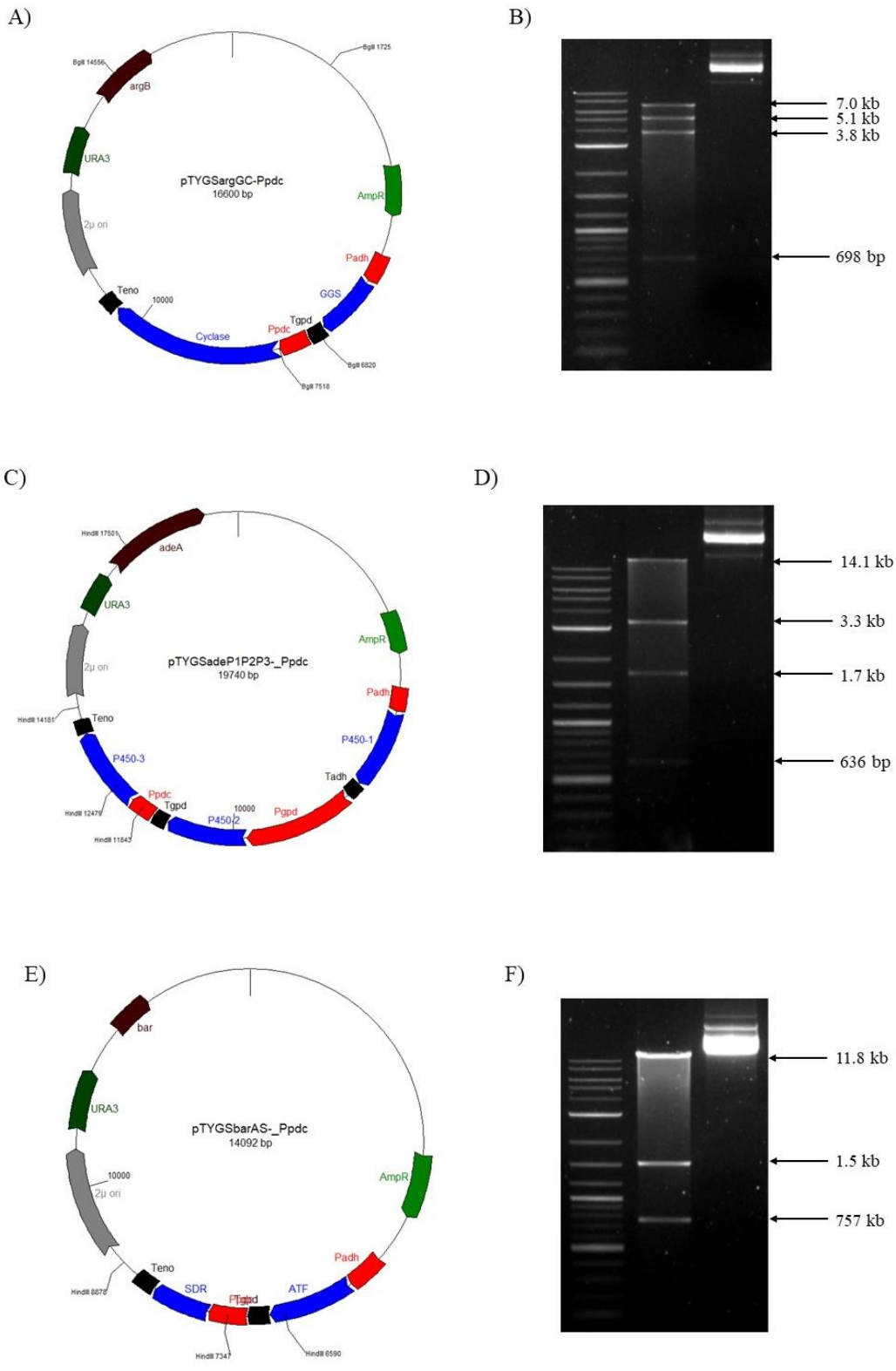


Figure 20 Predicted maps and confirming digests: Illustrating correct assembly of the new pleuromutilin plasmids with A-B) Cyclase, C-D) P450-3 and E-F) SDR under control of the *pdc* promoter. Lane 1 contains 5 µL of 2-log ladder. Lane 2 contains 10 µL of digestion reaction with restriction enzyme. Lane 3 contains 1 µL of undigested plasmid DNA.

3.3.6 *A. oryzae* transformation with pTYGSarg/GC-pdc

The *pdc* promoter was chosen to drive Cyclase expression which was previously under the control of the *eno* promoter. It was predicted that the *pdc* promoter would lead to the better expression of Cyclase gene, so expression of GGS together with pdc-Cylcase should provide a higher accumulation of the 3-deoxo-11-dihydroxy-mutilin (metabolite 1), the first intermediate of the pleuromutilin biosynthesis pathway.

The plasmid, pTYGSargGC-Ppdc was used to transform *A. oryzae* NSAR1 for heterologous expression. After subculture three times, seven stable transformant strains named as *A. oryzae* GC-Ppdc TS2- (1-7) were generated from the transformation. Successful transformation of pTYGSarg/GC-Ppdc was confirmed through PCR amplification for the presence of Ppdc-Cyclase (Figure 21). All the *A. oryzae* GC-Ppdc TS2- (1-7) transformant strains were positive for the presence of the Ppdc-Cyclase fragment.

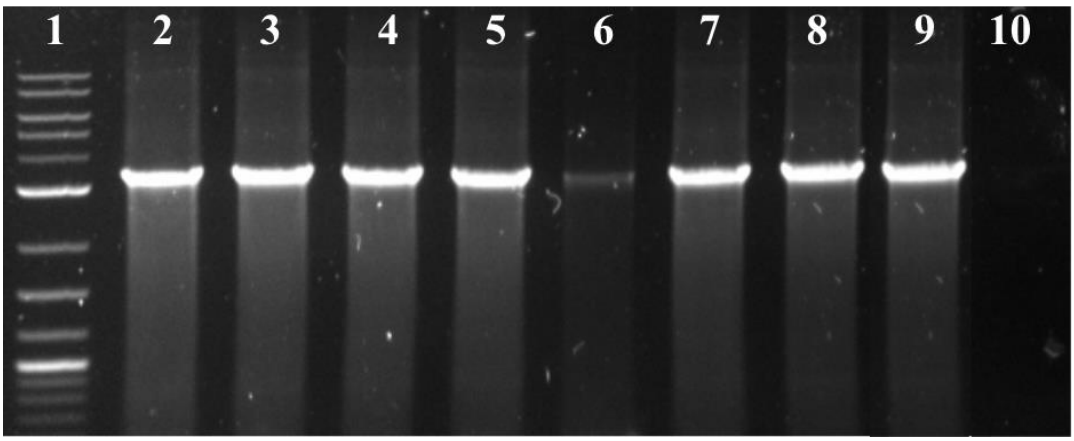


Figure 21 Gel electrophoresis showed PCR amplification of Ppdc-Cyclase (expected fragment 3380 bp) from gDNA extracted from *A. oryzae* GC-Ppdc transformant strains TS2-(1-7). Lane 1 contains 5 μL of 2-log ladder. Lane 2-8 gDNA of *A. oryzae* transformant strain was used as template. Lane 9 the same plasmid used for transformation was used as template. Lane 10 water was used as negative control.

3.3.6.1 Bioassay screening for antibiotic activity of *A. oryzae* GC-Ppdc genes transformant strains

Preliminary screening for antibacterial activity of the seven *A. oryzae* GC-Ppdc TS2- (1-7) were done against *B. subtilis*. The engineered strains were cultured separately on CMP agar plates for 3 days. Then TSAg containing *B. subtilis* and TTC was used to overlay around the fungal colony to develop a clearing zone to detect if any antibiotic was being produced. The plates were incubated overnight at 28^{o}C and the next day they were examined to see if any inhibition zone was present and zones were measured. Plate-based assay showed that *A. oryzae* GC-Ppdc TS2-2 had the strongest antibacterial activity with the largest clearing zone, while *A. oryzae* GC-Ppdc TS2- 4, 3 and 6 also demonstrated antibacterial activity. No clearing zone could be observed from *A. oryzae* GC-Ppdc TS2- 1, 5 and 7 (Table 6). Transformant TS2-2 only gave a zone of 2-3mm, so antibiotic activity was comparatively weak as would be expected from a pathway intermediate.

Table 6 Results of the bioassay screening for antibacterial activity of *A. oryzae* GC-Ppdc TS2- (1-7) against *B. subtilis*.

Sample	Results of the bioassay screening for antibacterial activity against *B. subtilis*
A. oryzae NSAR1	–
A. oryzae GC-Ppdc TS 2-1	–
A. oryzae GC-Ppdc TS 2-2	+
A. oryzae GC-Ppdc TS 2-3	+
A. oryzae GC-Ppdc TS 2-4	+
A. oryzae GC-Ppdc TS 2-5	–
A. oryzae GC-Ppdc TS 2-6	+
A. oryzae GC-Ppdc TS 2-7	–

3.3.6.2 Chemical analysis of *A. oryzae* strains containing pTYGSarg/GC-Ppdc for production of 3-deoxo-11-dehydroxymutilin (metabolite 1)

The three transformant strains of *A. oryzae* GC-Ppdc TS2- 2, 3 and 4 were progressed through chemical analysis. The spores of each transformant were cultured in 100 mL CMP for 10 days. The cultures were then extracted with ethyl acetate. After that the crude extracts were analysed for the presence of metabolite 1 to confirm the activity of GGS and Cyclase-Ppdc using Thin layer chromatography (Figure 22). It was shown that all three transformant strains were producing metabolite 1, so confirming the activity of GGS and Cyclase. In term of apparent yield, the spot of TS2-2 presented a slightly stronger accumulation of metabolite 1 than the others transformant strains, including TR5, where Cyclase was driven under *Peno*. Thus, it could be concluded that the expression of the Cyclase under the stronger *pdc* promoter does work and could provide better yield of metabolite 1.

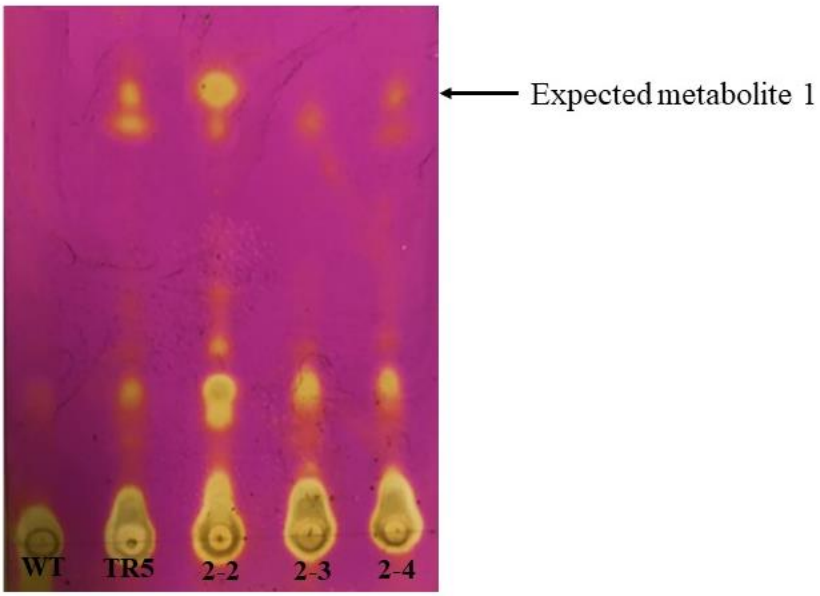


Figure 22 Thin layer chromatography demonstrated the presence of metabolite 1 from *A. oryzae* GC-Ppdc TS2- 2, 3 and 4 when compared with *A. oryzae* WT (WT) and *A. oryzae* GC TR5 which was the original strain where Cyclase was under the control of *Peno*.

3.3.7 *A. oryzae* GC-pdc transformation with pTYGSade/P1/P2/pdc-P3 (generation of 5 genes transformant strains)

From the previous transformation and chemical analysis, *A. oryzae* GC-pdc TS2-2 produced the most metabolite 1, so was the strain was chosen for the second round of transformation with pTYGSade/P1/P2/pdc-P3 plasmid**,** to create a five-gene transformant strain. Five independent transformant strains were generated from the second transformation and were named as *A. oryzae* GC-pdc/P1P2P3-pdc TS5- (1-5). The stable and correct transformants were tested by PCR amplification to assess the presence of Ppdc-P450-3 (Figure 23). All transformant strains were positive for the presence of the Ppdc-P450-3 fragment.

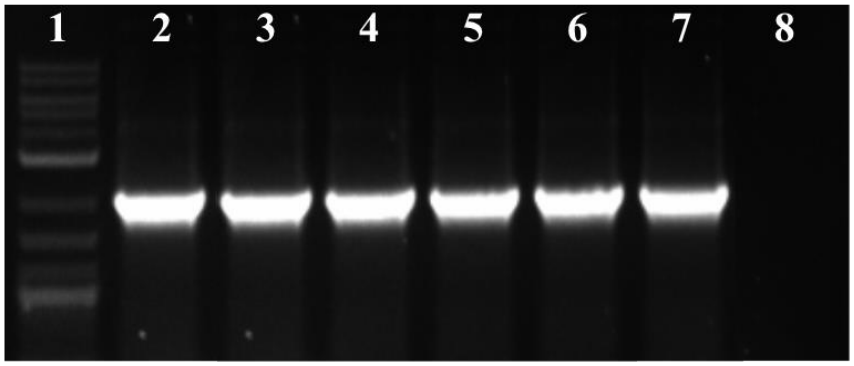


Figure 23 Gel electrophoresis of PCR amplification of Ppdc-P450-3 (expected fragment size of 2069 bp) from gDNA extracted from *A. oryzae* P1P2P3-Ppdc transformant strains TS5- (1-5). Lane 1 contains 5 μL of 2-log ladder. Lane 2-6 gDNA of *A. oryzae* transformant strain was used as template. Lane 7 the same plasmid used for transformation was used as template. Lane 8 water was used as negative control.

3.3.7.1 Bioassay screening for antibiotic activity of *A. oryzae* 5 genes transformant strains

The plate-based bioassay against *B. subtilis* was used to determine an antibiotic activity from *A. oryzae* 5-gene transformant strains. The assay was carried out by culturing each *A. oryzae* GC-pdc/P1P2P3-pdc TS5- (1-5) on CMP agar plates at 28^{o}C for 3 days. Then the transformant colonies were overlaid with TSAg containing *B. subtilis* and TTC. After overnight incubation at 28^{o}C, clearing zones were observed and measured to confirm an

antibiotic activity. The bioassay screening for antibiotic activity of *A. oryzae* GC-pdc/P1P2P3-pdc TS5- (1-5) showed that TS5- 1, 3, 4 and 5 had a weak antibiotic activity, while there was no clearing zone observed for TS5-2 (Table 7).

Table 7 Results of the plate-based antibiotic bioassay of *A. oryzae* 5-gene transformant strains against *Bacillus subtilis*.

Sample	Results of the plate-based bioassay against Bacillus subtilis to test antibiotic activity
A. oryzae NSAR1	–
A. oryzae GC-pdc/P1P2P3-pdc TS 5-1	+
A. oryzae GC-pdc/P1P2P3-pdc TS 5-2	–
A. oryzae GC-pdc/P1P2P3-pdc TS 5-3	+
A. oryzae GC-pdc/P1P2P3-pdc TS 5-4	+
A. oryzae GC-pdc/P1P2P3-pdc TS 5-5	+

3.3.7.2 Chemical analysis of *A. oryzae* 5 genes transformant strains for pleuromutilin

A. oryzae GC-pdc/P1P2P3-pdc TS5-3 and TS5-4 were chosen and progressed through chemical analysis for metabolite extraction. Spores of *A. oryzae* GC-pdc/P1P2P3-pdc TS5-3 and TS 5-4 were cultured in 100 mL CMP and incubated with shaking at 28^{o}C for 10 days. The culture was extracted with ethyl acetate and the crude extract was analysed by TLC for metabolite production. The TLC analysis of the extracts from *A. oryzae* TS5-3 and 5-4 (Figure 24) showed the accumulation of two candidate pleuromutilin intermediates. The first compound with high mobility, indicated in the upper red box, was suspected to be metabolite 1, the product of GGS and Cyclase. The second compound with lower mobility indicated in the lower red box was suspected to be either the 3-deoxo-mutilin (metabolite 2) resulting from the expression of GGS, Cyclase and P450-1 or the 3-dihydro-mutilin (metabolite 3), which would result from the expression of GGS, Cyclase, P450-1 and P450-2, or it could be a new metabolite. The results also illustrated the likely incomplete conversion of metabolite 1 to the next intermediate of the pathway. However, these results still support the hypothesis

that metabolite 1 structure was modified by the addition of the P450 genes. These genes are responsible for introducing hydroxyl groups to the pleuromutilin scaffold, resulting in a higher polarity of the molecule, so a compound with reduced mobility on TLC would be expected. Further characterisation of these two compounds was attempted but was unsuccessful.

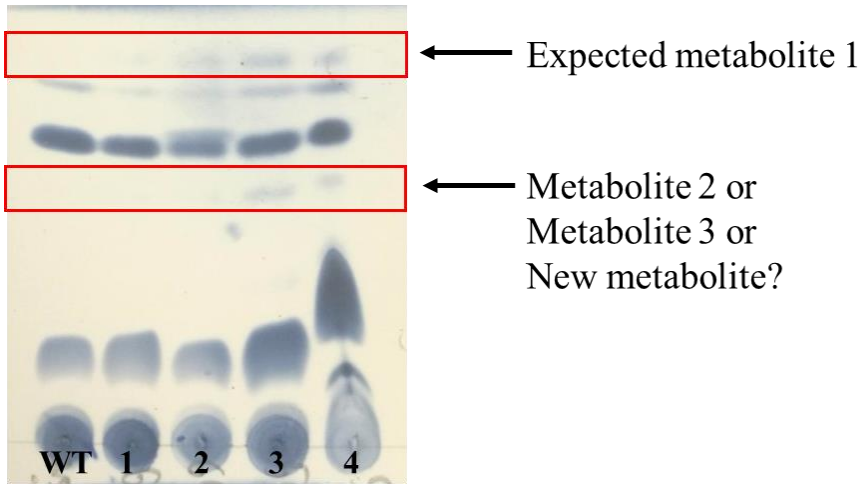


Figure 24 TLC analysis of the extract from *A. oryzae* transformant strains containing 5 genes showed the accumulation of suspected metabolite. From left to right; WT= *A. oryzae* NSAR1 wild type, 1 = *A. oryzae* GC-pdc strain TS2-2, 2 = *A. oryzae* GC-pdc strain TS2-3, 3 = *A. oryzae* GC-pdc/P1P2P3-pdc strain TS5-3 and 4 = *A. oryzae* GC-pdc/P1P2P3-pdc strain TS5-4.

3.3.8 Feeding experiment of *A. oryzae* strains contain pTYGSade/P1P2P3-pdc with 14-O-acetyl-mutilin

Khairudin (Khairudin, 2018) had previously shown that non-acetylated mutilin derivatives could be fed to *A. oryzae* expressing P450-3 and ATF and could be modified accordingly. However, the P450-3 step was often poor or incompletely achieved. Therefore, the new transformants with P450-3 under regulation of the *pdc* promoter were evaluated to see if they could overcome this problem.

A. oryzae transformants with P1P2P3-pdc TS3- (1-6) were cultured at 28ºC for 10 days in 100 mL CMP which was supplemented with 14-O-acetyl-mutilin, the synthetic intermediate kindly provided and synthesized by Jonathan Davies, School of Chemistry, University of Bristol. The cultures were chemical extracted with ethyl acetate, and crude extracts were analysed through HPLC for the production of pleuromutilin (Figure 25). The HPLC showed presence of the substrate in all samples. Transformants 2, 3 and 6 had a weak but detectable peak at 11.95 min characteristic for pleuromutilin, but the conversion was very inefficient despite having the *pdc* promoter.

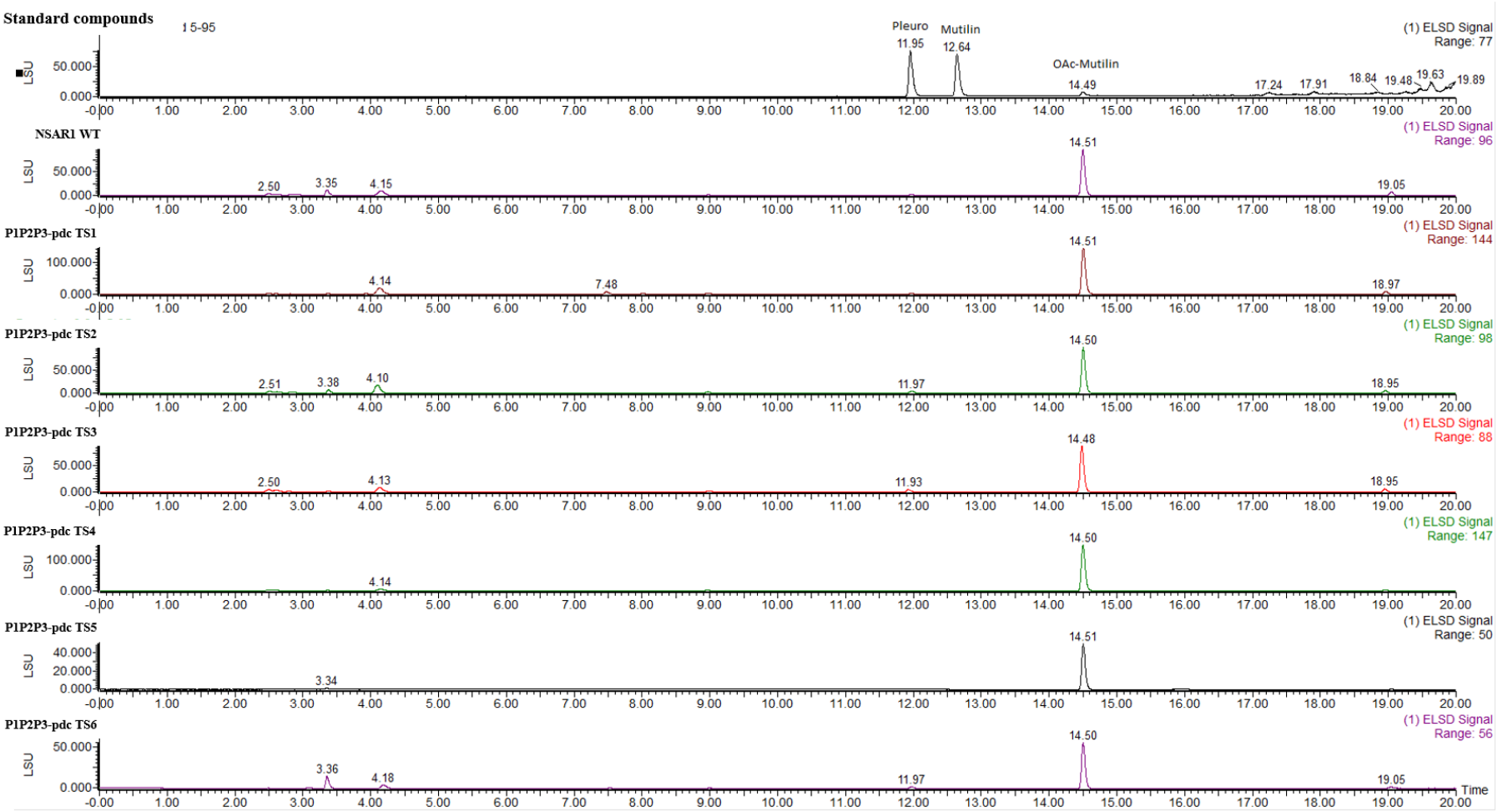


Figure 25 The ELSD chromatograms of standard compounds compared with *A. oryzae* WT and crude extracts from *A. oryzae* P1P2P3-pdc transformant strains, fed with 14-O-acetyl-mutilin. Little if any pleuromutilin was detected in the fed cultures.

3.4 Discussion

The work described in this chapter aimed to improve the pleuromutilin production by heterologous expression of the pleuromutilin biosynthesis pathway in the secondary fungal host, *Aspergillus oryzae*. The strategy was to enhance the expression level of the promoter that drive the genes within the cluster. In addition, I set out to assess whether synthetic compounds fed to *A. oryzae* transformant strain could be an efficient route to obtain new derivatives or compounds with more potent antibiotic activity.

The genes under the control of enolase promoter (*Peno*) were previously shown to have weak expression compared with other genes under the control of alcohol dehydrogenase (*Padh*) and glyceraldehyde-3-phosphate dehydrogenase (*Pgpd*) promoters (Alberti et al., 2017). Therefore, the low amount of one enzyme might result in the low level of other substrates and limit the final product to be obtain from the pathway. It was also hypothesised that the enzymes participating in pleuromutilin biosynthesis pathway might act as a multienzyme complex, which means that the product from one enzyme might channel directly to other enzymes along the pathway, and if one protein was in limiting amount, it might prevent correct assembly of the entire enzyme complex. To replace the enolase promoter with a stronger promoter is a hypothesis to improve the levels of gene expression and so overcome either of these limitations.

For this project, the candidate for a new promoter to replace *Peno* was chosen based on the expressed sequence tag (EST) database analysis of *A. oryzae* (Akao et al., 2007, Wang et al., 2010). Thiamine thiazole synthase (*ptrA*), phosphoglycerate kinase (*pgkA*) and pyruvate decarboxylase (*pdcA*) were the three promoters that were selected due to their plentiful expression in liquid culture compared to the enolase promoter (Wang et al., 2010). With the expectation that these promoters would have a great potential and strong activity in *A. oryzae* for the expression of a foreign gene, the investigation of each promoter's expression was done by assessing the GFP reporter gene expression. As a result, the *pdc* promoter showed the strongest expression of GFP from the mycelia of a stable *A. oryzae* transformant when observed under the fluorescence microscope. Similar results from the EST library also supported the high expression of *pdc* promoter. The cDNA library from *A. oryzae* mycelia was constructed, which each clone was made from 3' region of mRNA and the result showed that out of 345 randomly selected clones, *pdc* had the highest level of expression (Hwang et al., 1998, Lee et al., 1999, Liu et al., 2012).

Therefore, the *pdc* promoter was chosen to use as a new promoter to replace *Peno* and drive the genes in the pleuromutilin biosynthesis pathway. Yeast recombination was used as a method to replace *Peno* with *Ppdc*. Three new plasmids: pTYGSarg/GC-pdc, pTYGSade/P1P2P3-pdc and pTYGSbar/AS-pdc were constructed, the Cyclase, P450-3 and SDR genes were now under the control of the pdc promoter that was expected to deliver better expression. The first plasmid, pTYGSargGC-pdc, was made and transformed into *A. oryzae* NSAR1 WT. Although not fully quantified, TLC of crude extract from *A. oryzae* GC-Ppdc TS2-2 suggested an increase in production of metabolite 1 compared with the previous *A. oryzae* GC transformant used by Alberti et al., (2017). It is assumed that the expression of Cyclase was better under the control of *pdc* promoter although this was not quantified. Other report have also showed the potential of the *pdc* promoter for the expression of heterologous genes for example in *Zymomonas mobilis*, the resulted had shown that *ala*D gene was driven by the *pdc* promoter exhibited the double in expression than its natural *ala*D producer (Uhlenbusch et al., 1991). Unfortunately, the production of the expected metabolite 1 in this study was still limited given the expected strength of this promoter. Even though, the *pdc* promoter was shown to drive GFP effectively, it is worth highlighting that the 500 bp of the *pdc* promoter region used in this study might not include all of the necessary regulatory elements and perhaps a larger region would have supported stronger expression.

Progression towards the complete pleuromutilin structure was achieved by introducing the pTYGSade/P1/P2/P3-pdc vector into the *A. oryzae* GC-pdc T2-2. The chemical analysis from the extracts of the 5 gene transformants showed the accumulation of two metabolites. One was suspected to be metabolite 1. The other one was suspected to be either the known intermediate metabolites 2 or 3, or a new metabolite. In order to confirm identity of these two compounds, a more sensitive separation technique such as liquid chromatography-mass spectrometry (LC-MS) is needed for detection.

The final investigation was the feeding experiment to investigate the activity of P450-3 on fed substrates. By working together with the group from Chemistry, the *A. oryzae* transformant strains TS3- (1-6) containing pTYGSadeP1P2P3-Ppdc were use in feeding experiments with the synthetic intermediate 14-O-acetyl-mutilin and assessed for C22 hydroxylation via LC-MS analysis. The hypothesis of this strategy is that the better expression of P450-3 under the control of *pdc* promoter could lead to better ability of P450-3 to provide more effective conversion of 14-O-acetyl-mutilin to the final product,

pleuromutilin. A higher titre of pleuromutilin or finding a new compound that has a better antibiotic activity are also the expected result from this scheme. Unexpectedly, the conversion of 14-O-acetyl-mutilin to the final product, pleuromutilin by P450-3 under the control of *pdc* promoter was extremely poor. It had been predicted that good expression should result in better than 80% conversion (based on the approximately 50% conversion seen previously with the enolase promotor) and the reason for this lack of conversion is unclear. It should be noted that fewer than ten *A. oryzae* transformants were selected for chemical analysis in this study. Given that fungal transformation results in integration of the transforming DNA into the chromosomes, each transformant would likely have a different number of copies of the insertion, and at loci likely to give differing levels of expression. Therefore, the number of transformants being screened might not enough to find any effective production of an expected metabolites. To find a strain with a highly expressed gene to accumulate a compound, larger number of transformants would be needed to be screened. In order to get a more reliable result, around 20-30 positive transformants should be tested.

It is possible that the feeding experiment was unsuccessful due to the growth conditions and the experiment assumes that the substrate is taken up by the fungus. The components of growth media such as carbon and nitrogen sources seem to be a strong influence on the physiology of the fungus and thus whether it is in a growth phase amenable for production of secondary metabolites (Bose et al., 2019). In order to maximise metabolite production or indeed activity of enzymes such as P450s, optimisation of culture media and culture conditions should be performed.

Another potential concern is that basidiomycetes enzymes may not function properly in the ascomycete cytoplasm, so it is possible that the P450s were expressed efficiently, but were not being properly activated by the *Aspergillus* cytochrome P450 reductase (CPR) or perhaps not directed to the appropriate subcellular compartment (Nagamine et al., 2019). For a better understanding of what had happened, RNA-based analysis should be performed on the cDNA of each *A. oryzae* transformant strains to confirm efficient gene expression and perhaps accompany this with co-expression of the basidiomycete CPR to ensure the P450s are active.

3.5 Summary

In conclusion, heterologous expression of selected genes of the pleuromutilin biosynthesis pathway in *A. oryzae* transformants strains was performed after replacing the enolase promoter with a stronger promoter, pyruvate decarboxylase. This did not deliver a higher titre of pleuromutilin pathway metabolites. A more flexible heterologous host system might be another strategy to achieve a better expression of the pleuromutilin biosynthesis pathway.

Chapter 4. Feasibility of production of pleuromutilin *in planta* by recreating the pleuromutilin biosynthetic pathway in *N. tabacum*

4.1 Introduction

Plants are well known as producers of compounds in the terpene family. Many of the scents made by flowers are often terpene or sesquiterpene based, for instance sandalwood and lavender (Guitton et al., 2010, Jones et al., 2011). The active ingredients of many herbs and spices used in cooking are also terpenes, e.g. basil, oregano and hops (Steenackers et al., 2015, Yang et al., 2007). All higher plants have the ability to make diterpene compounds, for instance the phytohormone gibberellin is a diterpene, and is used in controlling cell expansion, involved in etiolation of shoots amongst other things (Hedden and Sponsel, 2015). From a medical perspective, there are also important plant-derived terpenes such as the sesquiterpene-based artemisinin, used in the treatment of malaria, produced by sweet wormwood *Artemisia annua* (Arsenault et al., 2008), or the diterpene taxol, a potent anti-cancer drug, derived from the leaves of *Taxus baccata*, the common yew tree (Heinig and Jennewein, 2009). Given that plants are clearly capable of generating commercial levels of useful terpenes, this chapter set out to investigate whether it was feasible to engineer production of pleuromutilin into plants. Plants, being comparatively easy to grow, provide readily scalable production platforms which should allow the cost-effective, timesaving and efficient expression of products compared to traditional fermentation-based methods that require expensive equipment and have high energy requirements both for sterilising media/waste and for continual operation. Moreover, when compared with the outcomes from animal or microbial culture, the products generated from plant system are safer (no risk of human-infecting viruses), easier to produce and less expensive (Fischer et al., 2004).

In recent years the ability to engineer plants has made some significant steps forward, mostly linked to engineering of crops. These typically only introduce one or two genes into the plant, often with protein products as their main aim and so are of limited use in engineering a multi-step biochemical pathway. Numerous studies demonstrated that various proteins with therapeutic use such as complex antibodies and vaccines, were produced from the feasibility and advantages of plant-based production platforms. (Orzaez et al., 2006, Virdi and Depicker, 2013). The release of “Golden rice” shows how engineering of novel metabolites can be

achieved in plants. Here rice was made to accumulate the yellow compound beta carotene, a precursor of vitamin A. This was achieved by heterologous expression of two genes, to convert host-derived geranylgeranyl pyrophosphate using phytoene synthase from daffodil and phytoene desaturase from the soil bacterium *Erwinia*, which, along with the hosts lycopene cyclase, gave low yields of beta carotene in the seeds (Ye et al., 2000). This was subsequently improved by (Paine et al., 2005) using the phytoene synthase from Maize giving higher yields.

4.1.1 Plant-based systems for heterologous expression

The majority of reports for plant biotechnology have required expression of just a few genes, which can be achieved using simple expression constructs. Even the example above of golden rice was only expression of two transgenes in addition to the selectable marker. The pleuromutilin pathway involves seven biosynthetic genes, all of which would be needed for successful expression (perhaps with the exception of GGS – although this is a pathway-specific version in fungi). At the commencement of this project there were very few plant expression systems that would allow for easy construction of a seven-gene expression cassette.

Assembly of multi-component constructs such as promoter, gene of interest and terminator expression cassettes is a general task for molecular research. Many methods have been developed and used over the years. Often each approach will work only for a particular cloning project due to time limitation, cost, availability of materials, size of construction and specific preferences of the project.

Restriction enzyme cloning is a classic cloning method and remains one of the most general processes in everyday research. Based on digestion and ligation reaction by utilising enzymes that cut double stranded DNA into fragments which containing precise 5’ or 3’ single strand overhangs (sticky ends) or no overhang (blunt ends) in digestion reaction. The compatible DNA fragments that have complementary sticky or blunt ends then will be joined together in ligation reaction. This method is helped by the number of available enzymes, which also work for a specific target sequence, give a fully predictable result and also the price is relatively cheap. Unfortunately, it is a very time-consuming method and is not well suited for assembling a large number of fragments especially if the same region is present in several

places in the vector and also increasing the difficulty on an issue for PCR-based or yeast-based assembly (Emami et al., 2013).

Another popular recombination-based cloning exploiting the lambda phage's integration and excision mechanism, which was invented and commercialised by Invitrogen as Gateway® Recombination Cloning. This strategy is done via a single recombination reaction, simplifying the process and reducing the time compared to restriction enzyme-based cloning. In brief, the fragment of DNA to be joined needs to be amplified and flanked by specific recombination sites, Gateway attB1, and attB2. The PCR amplification product then is mixed with a donor plasmid by the BP clonase enzyme mix to create an entry clone. The entry clone cassette can then be easily shuttled into any compatible Gateway® Destination vector through LP clonase enzyme mix (Figure 26). A typical Gateway destination vector system for gene expression contains multiple cloning sites, a variety of promoters and different selection cassettes, allowing the insertion of genes of interest. The version called MultiSite Gateway® allows the cloning of up to five different fragments. However, there are several drawbacks of the method; the cloning kit is rather high cost, the recombination site leaves so-called scars with 25 base pair of unwanted sequence that might be a problem for expression and even with the MultiSite version, the number of fragments is limited to five. Therefore, it is not a suitable approach for assembling the pleuromutilin gene cluster in this project (Lampropoulos et al., 2013).

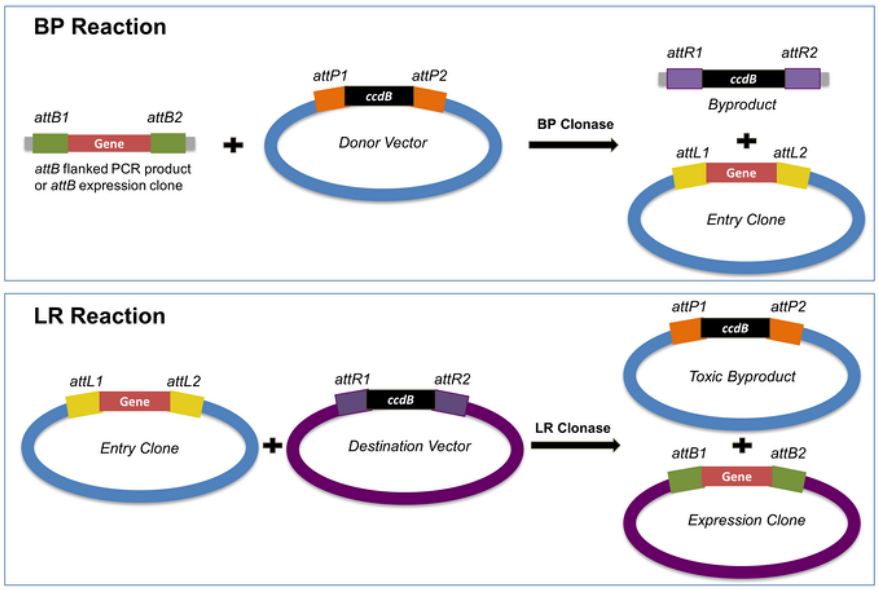


Figure 26 Illustration of the Gateway cloning process. Image from Soriano, (2017).

The Gibson cloning method, also known as Isothermal cloning (Gibson, 2011), takes advantage of the T5 exonuclease enzyme property, which digests a double stranded DNA in the 5'-3' direction to generate a DNA fragment with 3' single-stranded overhangs. DNA fragments that have 20-40bp of homology sequence at their ends are joined together (Figure 27). The process is similar to restriction enzymes cloning but with greater in length of complementary sequence and no need to introduce cloning scars. Phusion DNA polymerase is used to fill in any remaining regions between the annealed DNA fragments and then the ligase seals the nicks (Gibson et al., 2009).

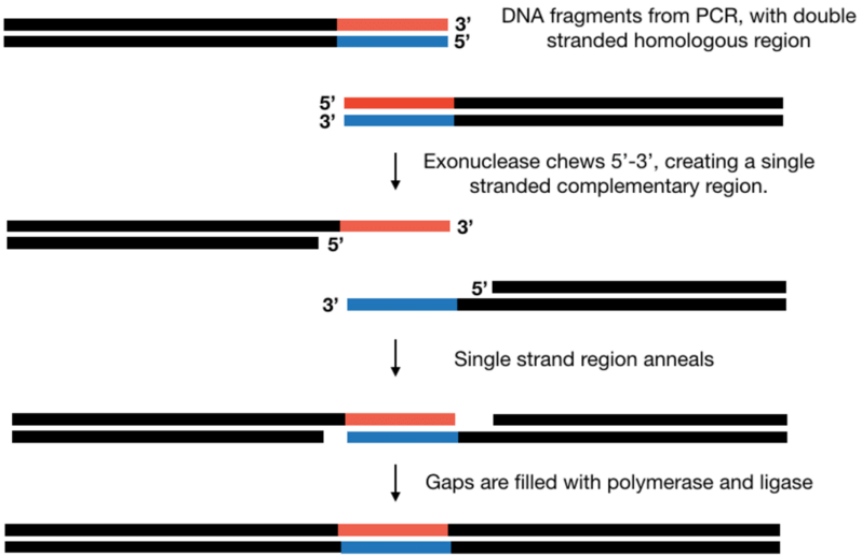


Figure 27 Outline of the Gibson cloning method. Image from Cutts and Vannini, (2018).

The advantages of this method are: 1) it is fast, flexible and lower in cost compared with commercial systems such as Gateway®, 2) it allows for multiple fragments of DNA to be combined, typically up to five fragments in a single reaction or up to fifteen fragments in two steps, 3) DNA fragments can be joined without creating any unwanted DNA sequence at the junctions when compared with restriction enzyme or Gateway recombination cloning. However, this method also contains a number of weaknesses: 1) undesired side products can be produced due to the repeats of homologous region used to anneal DNA fragments, 2) a stable secondary structure of single-stranded DNA such as hair-pins will not be able to base pair with its target, 3) small fragments less than 250 base pairs might be completely digested by the exonuclease enzyme before the annealing process (Emami et al., 2013).

The Golden Gate cloning system is a method that provides an efficient assembly of a large numbers of DNA fragments and the whole process is claimed to be faster and easier compared to the other methods mentioned above. Based on a unique action of type IIS restriction endonuclease enzymes, which cleave DNA at a site outside of their recognition sites, this results in DNA fragments that have compatible four bases overhang that can be ligated scarlessly. This method provided a feasible scheme for directional assembly of multiple fragments. According to Weber et al., (2011), 11 transcription units made from 44 basic fragments were successfully assembled and generated 33 kb construct via three successive one-pot cloning steps. Since the goal of this chapter is to clone the total pleuromutilin biosynthesis pathway into one expression vector, the Golden Gate cloning system appeared to be suitable to assemble multiple fragments, so was chosen as a tool to create the pleuromutilin expression vector for *in planta* use for this project. More detail of the Golden Gate cloning system will be described later in the results section of this chapter.

The choice of plant species for the expression of heterologous genes is an important factor in the design of a plant-based production system. The selection of plant host depends on several criteria; the nature of secondary metabolite, available technology for gene transfer and expression, the ability for regeneration and post-translational modifications in the host, ability to scale-up production, cost for maintenance and the downstream processing requirements (Makhzoum et al., 2014). There are variety of plant crops types that have been tested and used for the commercial production of pharmaceutical proteins such as leafy crops, seed and legumes crops, oil crops and fruit and vegetable crops.

Seed-based systems offer many advantages for example: providing a likelihood of long-term storage even at room temperature with no detectable loss of protein content, specific expression of transgene in seed has biosafety benefits as it will not affect other growth phases. However, the transgenic plants need to go through a flowering cycle to produce seed. This has issues for gene contamination and a chance of contamination through pollen transfer (Makhzoum et al., 2014, Rademacher et al., 2009). Several fruit and vegetable crops are being utilised for the production of recombinant vaccines and antibodies application, due to their advantage as edible plants that can be consumed directly. For instance, potatoes were chosen as a production system of rotavirus VP6 capsid protein to treat acute viral gastroenteritis (Yu and Langridge, 2003). But this platform is limited by the timing of ripening and harvesting, so production is seasonally dependent.

Leafy crops have major advantages in term of biomass yield, case of leaf harvesting that can be done without having a flowering cycle and thus also reduce contamination through pollen dispersal. Tobacco is one of the strongest candidates. It is a non-food crop, which will reduce the risk of contamination within human food chain (Twyman et al., 2003), it has high biomass yield, fast growth and easy harvesting all year round. It is ideal for transgene expression due to its long history as a plant-based system in molecular studies, gene transfer and expression methods are well-established and a large-scale infrastructure for manufacturing already exists (Makhzoum et al., 2014). One disadvantage of tobacco is that it contains high levels of phenolic compounds, which may need to be removed as part of the purification process.

4.2 Aims

The aim of the work described in this chapter was to recreate the pleuromutilin biosynthesis pathway in tobacco plants.

This could be broken down into several stages:

1. Choice of expression system to recreate the pleuromutilin biosynthesis pathway in tobacco plants
2. Making the plasmid construct containing the genes involved in the pleuromutilin biosynthesis pathway
3. *Agrobacterium* infiltration to generate transient *N. tabacum* plants expressing the pleuromutilin biosynthesis pathway to validate the pathway
4. Evaluate the phenotype development of infiltrated *N. tabacum*. If successful with transient expression, to progress through stable transgenic lines

4.3 Results

4.3.1 Rationale for the choice of vector system and design of the expression construct

To build an expression vector containing the seven genes involved in pleuromutilin biosynthesis and transform this into the secondary host, *N. tabacum*, various possible strategies were considered.

Yeast-based methods were a possibility using the yeast-adapted pCambia plasmids developed within the group, however the existing vectors were made for fungal transformation and there were not enough different plant promoters and terminators that were already fully validated to be able to assemble all the genes in one construct. It would be possible to split them across two or three plasmids providing enough plant selectable markers were available, or to transform different plants with each, and then cross them to get the constructs into the one plant, however, this was considered to be overly time-consuming.

A review of the literature identified that several technologies have been developed to allow assembly of multiple parts for biosynthetic pathway engineering in plants. For instance, the published Gateway methods allowed a few genes to be moved, but at the time of experimental design these were not readily available with plant expression cassettes and plant selectable markers in *Agrobacterium*-adapted systems, certainly not for the number of genes that needed to be co-expressed (Engler et al., 2008).

The Golden Gate cloning method was utilised to create a 50 kb construct that contained 17 transcriptional units (Werner et al., 2012). The construct was generated from assembling 68 DNA fragments using three rounds of one-pot Golden Gate cloning. In a study by Celińska and colleagues (Celińska et al., 2017), the Golden Gate system was employed to construct a complex vector for expression of the carotenoid synthesis pathway. This consisted of 12 basic DNA fragments (promoter, gene of interest and terminator). The final expression vector was generated from the assembly of three transcription units and was then used to transform into competent *Yarrowia lipolytica* cells to investigate for the carotenoid production, showing that terpene pathways are amenable to this expression system.

For this study, the Golden Gate cloning technology looked most suitable and so was chosen as a strategy to build a final expression vector containing the seven genes involved in the pleuromutilin biosynthesis pathway.

4.3.2 The principles behind Golden Gate assembly

Golden Gate assembly is based on the use of type IIS restriction enzymes (Engler et al., 2014). This group of enzymes recognize non-palindromic target sequences and cleave outside of their recognition site (Figure 28a) resulting in DNA overhangs that consist of specific unique sequences, allowing controlled, ordered assembly. By this process, DNA fragments flanked by compatible sequence overhangs could theoretically be ligated seamlessly. Furthermore, since the overhangs can be designed individually, the directional assembly of multiple fragments is feasible. This technology is claimed to be a powerful tool that allows highly efficient directional assembly of multiple DNA parts in a one-pot, one-step reaction (Patron et al., 2015, Weber et al., 2011) (Figure 28b).

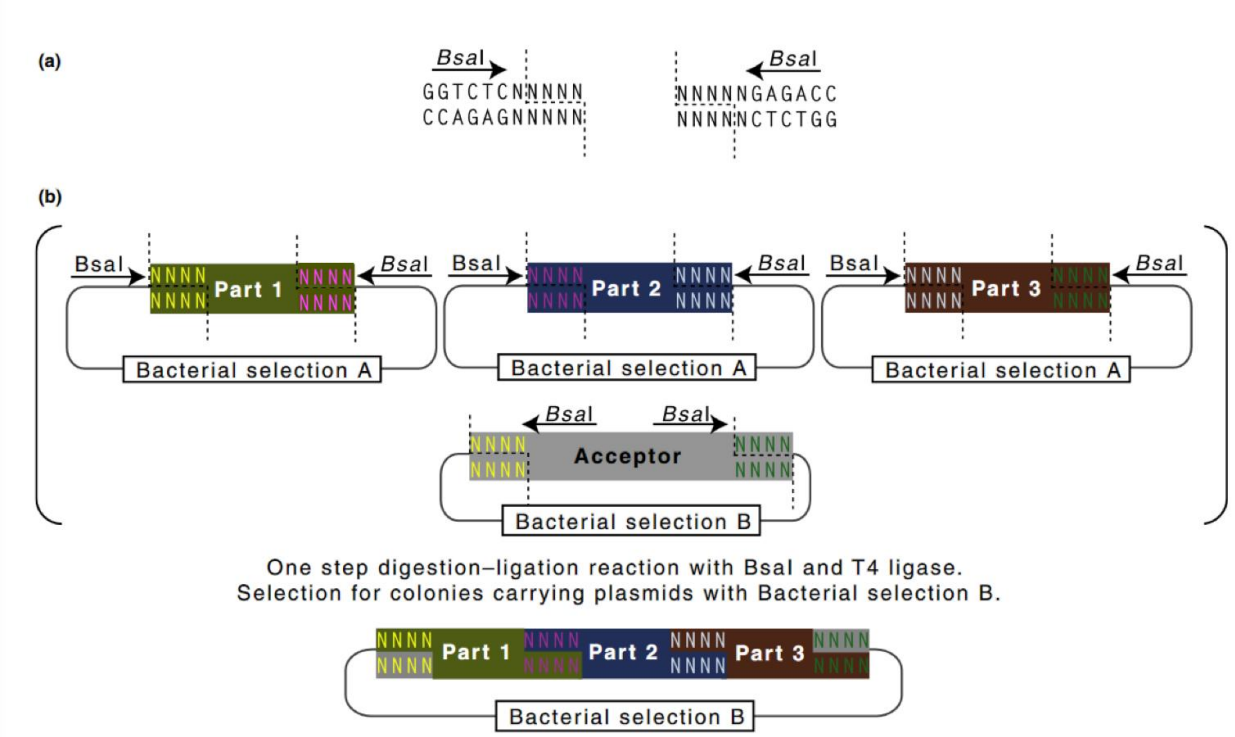


Figure 28 Outline of the generic Golden Gate assembly process. (a) The type IIS restriction enzyme *Bsa*I (or *Eco*31I) cleaves outside its recognition site and leaves unique 4-base overhangs. (b) Standard parts are cloned into plasmid backbones flanked by a pair of type IIS restriction enzyme recognition sites, and then all standard parts can be assembled into an acceptor plasmid flanked by compatible type IIS restriction enzyme recognition sites via a single digestion-ligation reaction. Image from Patron et al., (2015).

The Golden Gate process is split into several discrete steps. First each individual element is constructed, modified where necessary to ensure that they do not contain internal recognition sites for the various type II enzymes used in the process. These are termed level -1 fragments. These are then modified so as to have the appropriate restriction sites at each end, (level 0 plasmids). These elements are joined together to form each separate expression cassette (level 1 constructs) and these transcription units are then assembled, along with appropriate selectable markers or visual reporters either in one go, or stepwise, to deliver the final multi-gene plasmid (level 2 plasmids). The different levels of plasmid each use different antibiotic resistance markers to allow easy selection of desired recombinants, (Figure 29). In this way, at least in theory, it should be possible to put at least 6 transcription units into one plasmid in one single step (Weber et al., 2011).

Figure 29 Illustration of the various levels within the Golden Gate Assembly Standard Protocol. Image from Engler et al., (2014).

4.3.3 Module generation: the level -1 for removing internal type IIS enzyme target sites

Basidiomycete genes typically contain numerous introns, so the plan was to express cDNA, as fungal introns likely would not splice correctly in plants. Close inspection of the various pleuromutilin gene cDNA sequences showed that six out of seven cDNAs contain several internal sites for *Eco*31I or *Bpi*I, which would interfere with later steps in the vector construction and needed to be removed (Table 8).

Table 8 The number of internal type IIS recognition sequences for each cDNA.

GENE	NUMBER OF TYPE IIS RECOGNITION SEQUENCES NEEDING TO BE REMOVED
GGS	3
CYCLASE	8
P450-1	7
P450-2	8
P450-3	4
ATF	0
SDR	1

The strategy to remove these sites is shown in Figure 30. Primers were designed overlapping the internal *Eco*31I or *Bpi*I sites but containing a single nucleotide mismatch to eliminate the internal recognition sequence of *Eco*31I or *Bpi*I. All PCR fragments could then be joined together using yeast homologous recombination along with the pYES2 backbone plasmid which had been previously digested with the restriction enzyme *EcoR*I. Fragments to be inserted into each backbone plasmid were amplified through PCR with Phusion High-fidelity DNA polymerase with specific primers that introduce a single nucleotide mismatch where needed. The modifications were designed to be silent mutations so no changes would be made to the encoded protein.

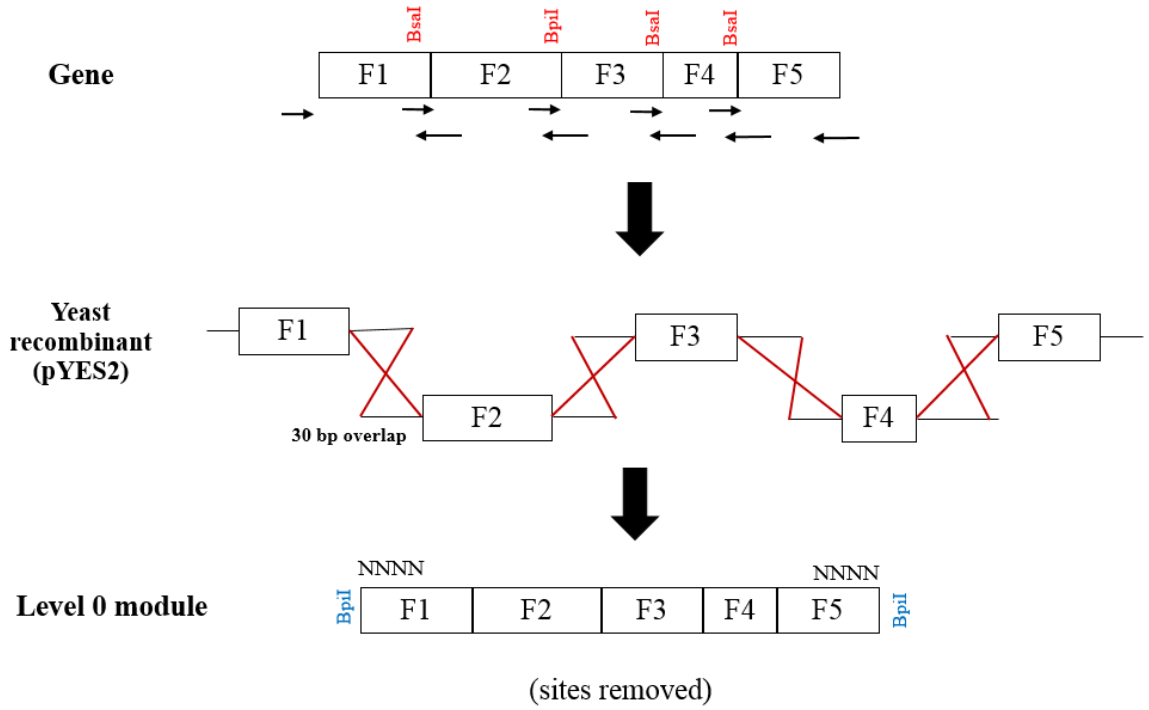


Figure 30 The generic strategy to remove internal type IIS recognition sequences from the cDNA. To remove the internal *Bpi*I and *Eco*31I sites within a fragment of gene, the specific primers were designed for amplifying between each site. The primers were designed to introduce a single nucleotide mismatch, so that the *Bpi*I and *Eco*31I internal sites would be eliminated and the fragments could be assembled via yeast homologous recombination.

Correct construction of pYES2/modified(m) of each cDNA was confirmed with PCR amplification of the desired gene (representative examples are shown in Figure 31-32. In addition, correct construction was also confirmed with restriction digest with the *Bpi*I and *Eco*31I enzymes showing removal of the unwanted sites and with sequencing to confirm cDNA were error-free. Eventually this was successfully performed for all of the cDNA, except P450-2, giving level-1 products ready for future manipulation.

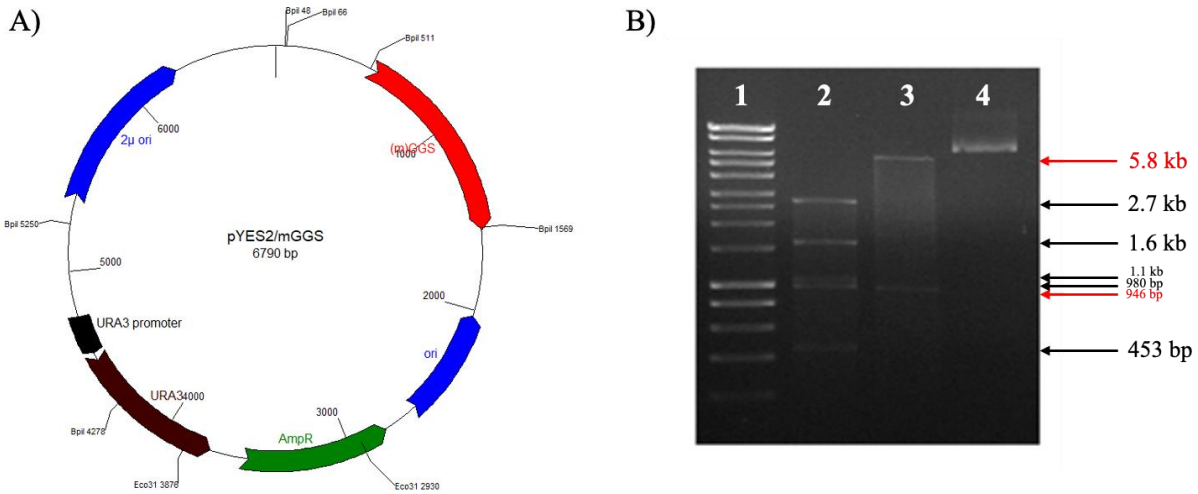


Figure 31 A) The predicted map for pYES2/mGGS without internal *Eco*31I and *Bpi*I recognition sequences. B) Restriction digest showing correct construction of plasmid pYES2/mGGS, with loss of the restriction sites from the GGS cDNA: lane 1 contains 5 µL of hyperladder I (Bioline), lane 2 contains 10 µL of digestion reaction with restriction enzyme *Bpi*I of pYES2/mGGS (expected fragments 2.7 kb, 1.6 kb, 1.1 kb, 980 bp, 453 bp and 10 bp), lane 3 contains 10 µL of digestion reaction with restriction enzyme *Eco*31I of pYES2/mGGS (expected fragments 5.8 kb and 946 bp were indicated in red) and lane 4 contains 1 µL of undigested plasmid DNA of pYES2/mGGS.

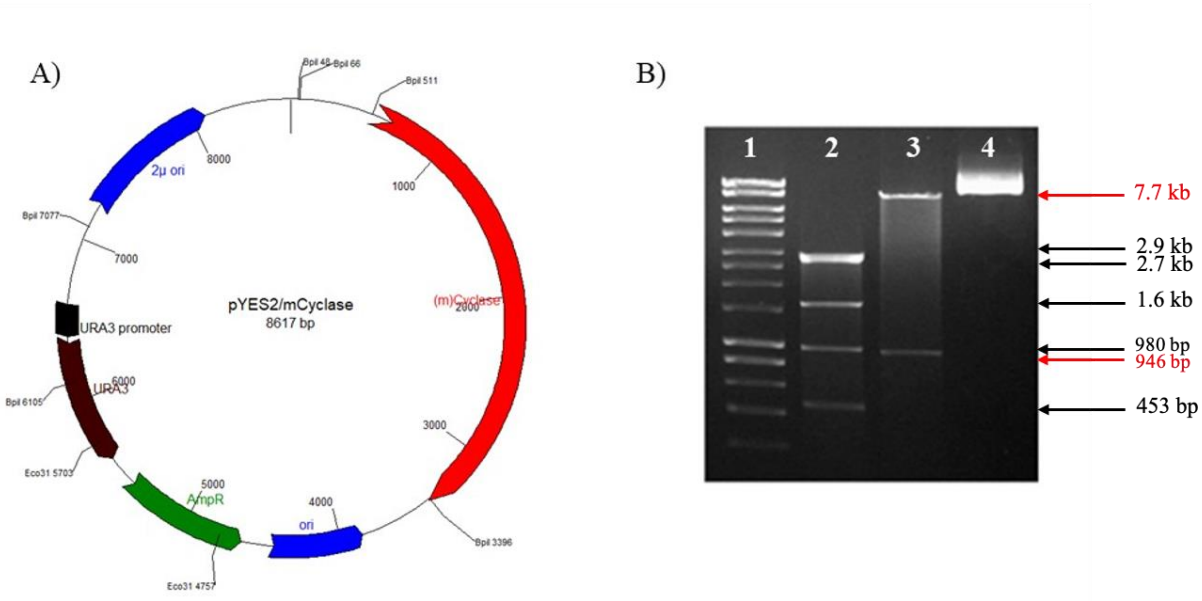


Figure 32 A) Predicted map of pYES2/mCyclase vector contains Cyclase gene without internal *Eco*31I and *Bpi*I recognition sequences. B) Restriction digest showing correct construction of plasmid pYES2/mCyclase, with loss of the restriction sites from the Cyclase cDNA : lane 1 contains 5 µL of hyperladder I (Bioline), lane 2 contains 10 µL of digestion reaction with restriction enzyme *Bpi*I of pYES2/mCyclase (expected fragments 2.9 kb, 2.7 kb, 1.6 kb, 980 bp, 453 bp and 10 bp), lane 3 contains 10 µL of digestion reaction with restriction enzyme *Eco*31I of pYES2/mCyclase (expected fragments 7.7 kb and 946 bp were indicated in red) and lane 4 contains 1 µL of undigested plasmid DNA of pYES2/mCyclase.

Unlike the other six genes, P450-2 contains the highest number of *Bpi*I and *Eco*31I internal sites. Moreover, the internal sites were close to each other, In order to recreate the site-free cDNA by yeast recombination, the rich internal sites region was divided into several very small fragments before assembling. These small fragments made it more difficult to purify the PCR products and also more challenging to assembly multiple fragments in yeast. After a number of failures to assemble the site-free cDNA by yeast recombination, the cDNA for P450-2 was made as synthetic DNA by vectorbuilder (Figure 33).

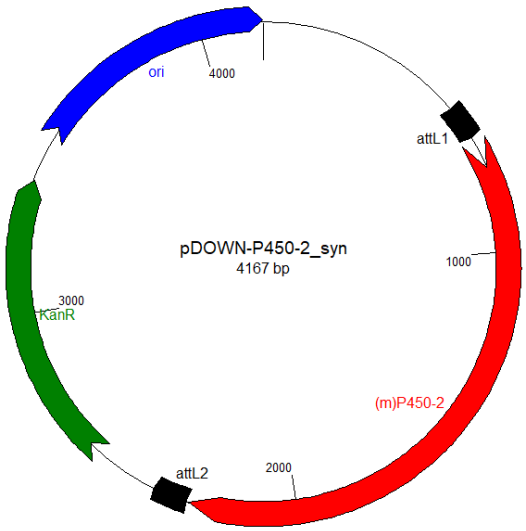


Figure 33 Plasmid map of the synthetic pDOWN/P450-2.

Having confirmed removal of the unwanted restriction sites from all the individual genes, the next step was construction of Golden Gate level 0 vectors.

4.3.4 Module generation: the level 0

For construction of the level 0 modules, the sequences of the seven pleuromutilin genes from pYES2-based level-1 vectors and nptII gene from pCAMBIA2300 were each PCR-amplified using Phusion High-fidelity DNA polymerase, adding the desired fusion sites (four unique nucleotides of choice which complementary to allow annealing to the end of next fragment or the vector) and *Bpi*I recognition site as part of the primers used for amplification, digested with *Bpi*I and each cloned into the L0 acceptor plasmid pAGM9121 (Figure 34) via a Golden Gate cloning reaction, leading to creation of a level 0 module of each gene: pL0-GGS, pL0-Cyclase, pL0-nptII, pL0-P450-1, pL0-P450-2, pL0-P450-3, pL0-ATF and pL0-SDR.

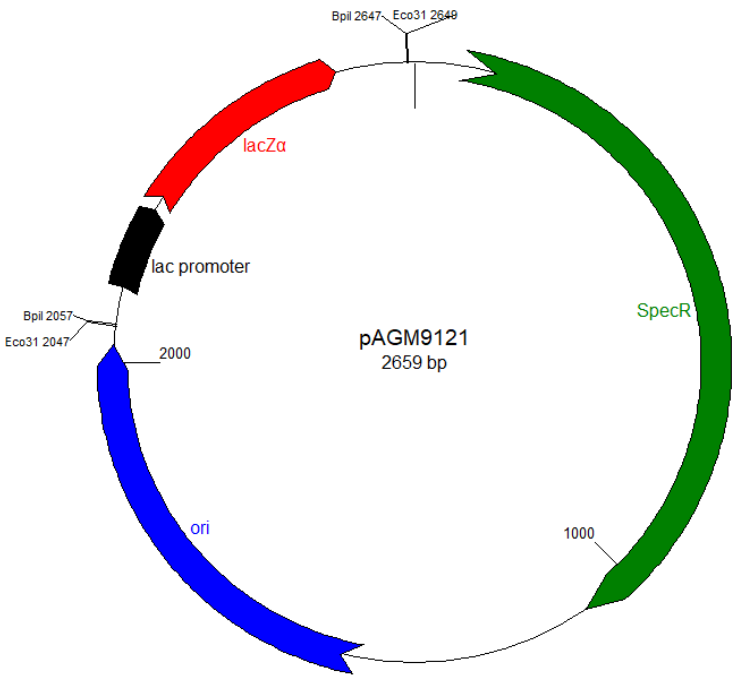


Figure 34 The level 0 destination vector, pAGM9121 (pL0), confers spectinomycin resistance (SpecR) and encodes a lacZα fragment for blue/white selection. Two different type IIS recognition sequences -*Eco31*I and *Bpi*I are flanked on both sides of the lacZα fragment in inverse orientation relative to each other. The DNA fragment of interest can efficiently clone into the vector via *Bpi*I. After removing the *Bpi*I recognition sites and lacZα, this design also provides further possibility to release the cloned fragment via *Eco31*I.

The successful correct construction of pL0- of each of the seven genes was confirmed with restriction digest with the appropriate enzyme and PCR amplification of the genes inserted. Examples for GGS and Cyclase are shown in Figure 35 and 36.

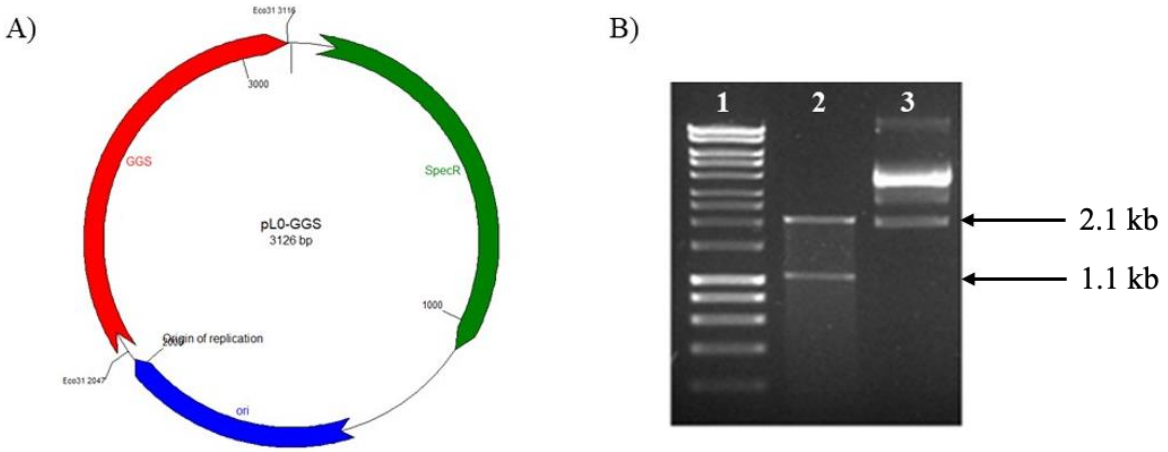


Figure 35 A) Predicted map of the pL0-GGS. B) Restriction digest showing correct construction of plasmid pL0-GGS: lane 1 contains 5 µL of hyperladder I (Bioline), lane 2 contains 1 µg of digestion reaction with restriction enzyme *Eco*31I of pL0-GGS (sites shown in green, expected fragments 2.1 kb and 1.1 kb) and lane 3 contains 1 µL of undigested plasmid DNA of pL0-GGS.

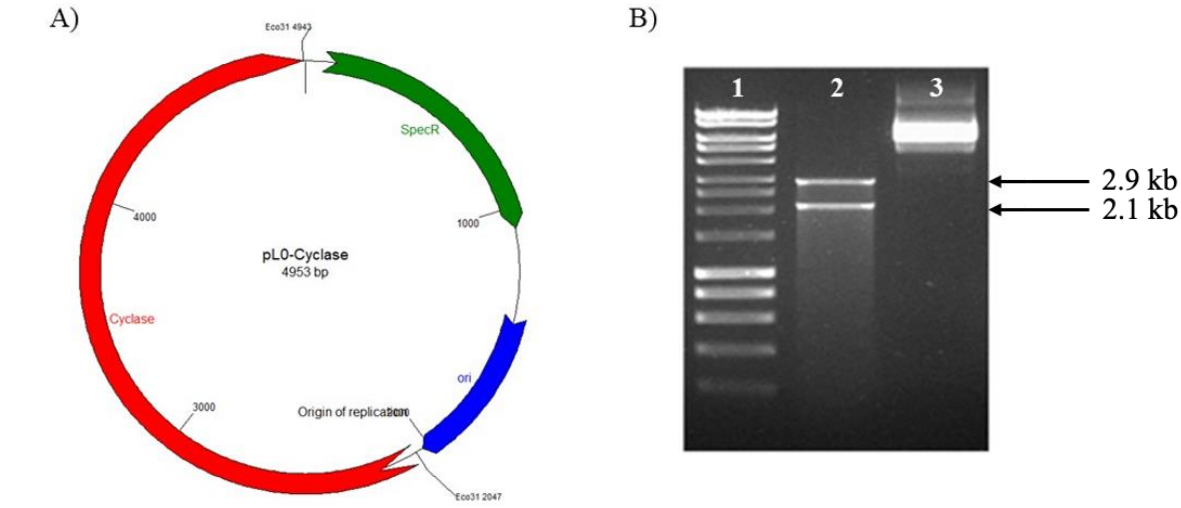


Figure 36 A) Plasmid map of the pL0-Cyclase. B) Restriction digest showing correct construction of plasmid pL0-Cyclase: lane 1 contains 5 µL of hyperladder I (Bioline), lane 2 contains 10 µL of digestion reaction with restriction enzyme *Eco*31I of pL0-Cyclase (expected fragments 2.9 kb and 2.1 kb) and lane 3 contains 1 µL of undigested plasmid DNA of pL0-Cyclase.

It was noted that the Golden Gate assembly was far less efficient than expected, and was rather variable (Table 9), but in each case a level-0 plasmid was eventually successfully constructed.

Table 9 Overall success rate in generating Level-0 plasmids for each gene.

Plasmid	Number of correct constructions
pL0/GGS	1/10
pL0/Cyclase	1/20
pL0/nptII	1/10
pL0/P450-1	1/10
pL0/P450-2	5/10
pL0/P450-3	4/5
pL0/ATF	3/5
pL0/SDR	2/5

4.3.5 Module generation: the level 1 to create a transcriptional unit

Compatible sets of sequenced level 0 modules (35S promoter, gene of interest, and nos terminator fragments) were then assembled into a level 1 vector with a second Golden Gate reaction using the enzyme *Eco*31I as outlined in Figure 37, leading to creation of a level 1 module which is referred to as a transcription unit (TU). Each TU contains the promoter, gene of interest and terminator. The level 1 destination vectors confer ampicillin resistance in order to allow the efficient counter selection against level 0 backbones. Similar to the level 0 destination vectors, two different type IIS recognition sites are also positioned on each side of lacZα fragment. This was performed for each of the eight required genes, generating pL1-GGS, pL1-Cyclase, pL1-nptII, pL1-P450-1, pL1-P450-2, pL1-P450-3, pL1-ATF and pL1-SDR.

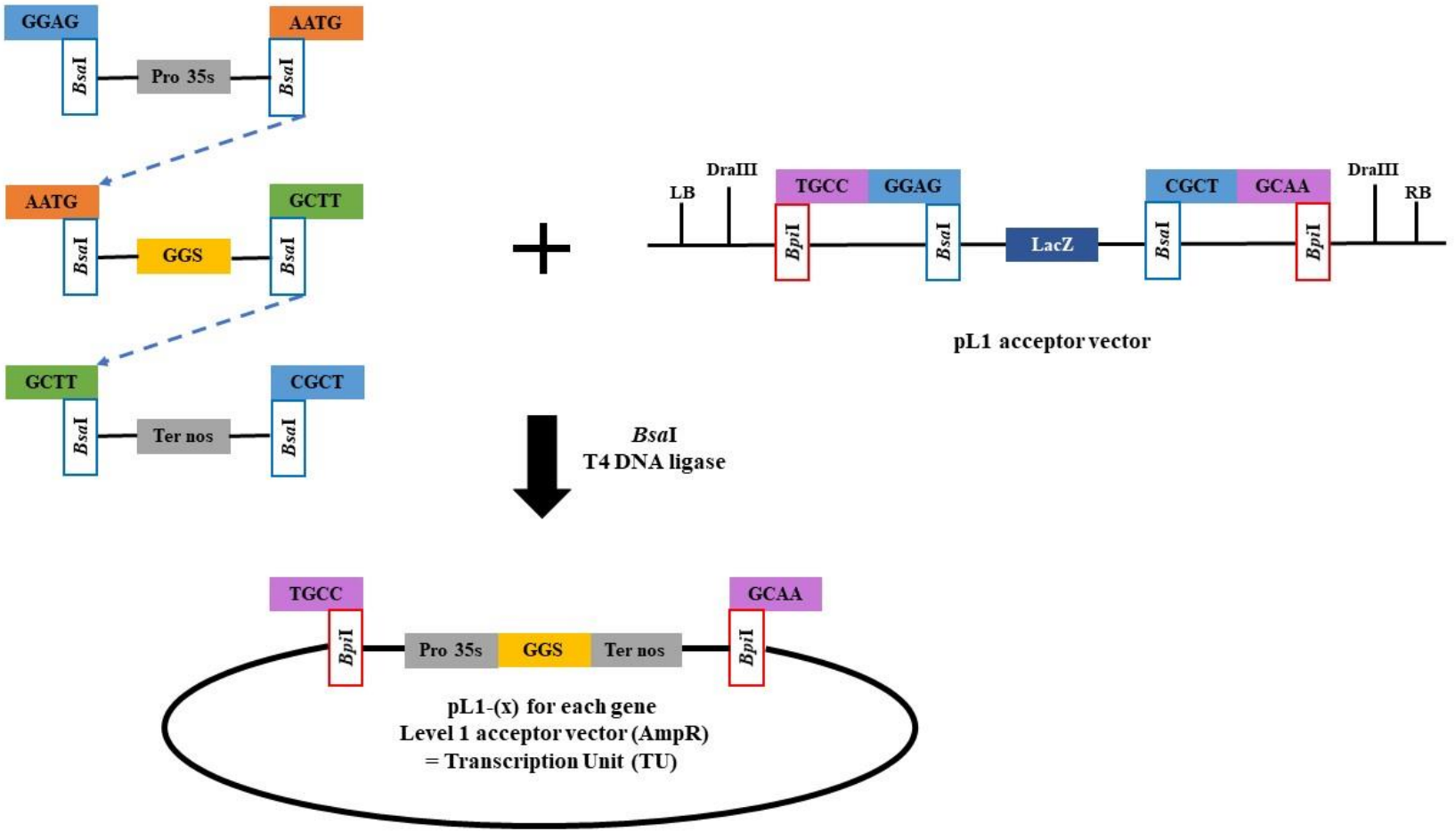


Figure 37 A scheme for the construction of pL1 plasmids. The compatible set of promoter, gene and terminator was assembled via *Eco*31I enzyme of the Golden Gate assembly reaction. As a result, transcription unit of gene is created and hanging with *Bpi*I on both sides, which is ready for the final assembly in level 2.

Correct construction of pL1- plasmid of each gene was confirmed with PCR amplification of the genes inserted. Restriction digests with *Bpi*I were done in order to confirm the completed pL1 plasmids that were ready for the final assembly in level 2. Examples are shown for GGS and Cyclase in Figure 38 and 39.

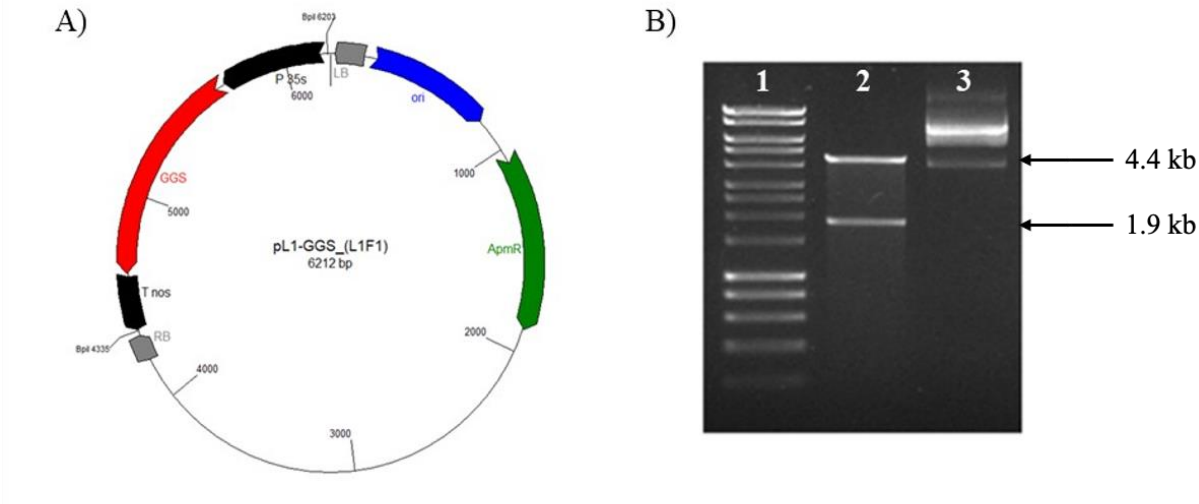


Figure 38 A) Plasmid map of the expression vector pL1-GGS. B) Restriction digest showing correct construction of plasmid pL1-GGS: lane 1 contains 5 µL of hyperladder I (Bioline), lane 2 contains 10 µL of digestion reaction with restriction enzyme *Bpi*I of pL1-GGS (expected fragments 4.4 kb and 1.9 kb) and lane 3 contains 1 µL of undigested plasmid DNA of pL1-GGS.

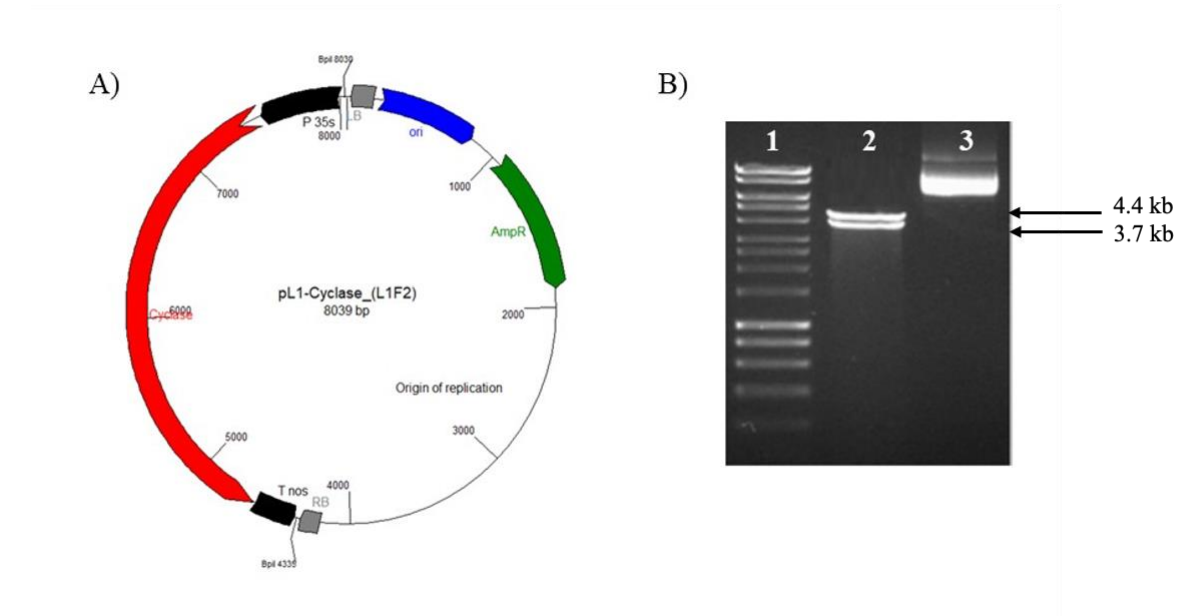


Figure 39 A) Plasmid map of the expression vector pL1-Cyclase. B) Restriction digest showing correct construction of plasmid pL1-Cyclase: lane 1 contains 5 µL of hyperladder I (Bioline), lane 2 contains 10 µL of digestion reaction with restriction enzyme *Bpi*I of pL1-Cyclase (expected fragments 4.4 kb and 3.7 kb) and lane 3 contains 1 µL of undigested plasmid DNA of pL1-Cyclase.

As with construction of the level 0 plasmids, the success rate was variable and low for this reaction, but candidate level 1 cassettes were eventually constructed for all eight genes (Table 10).

Table 10 The proportion of successfully completed pL1 plasmids.

Plasmid	Number of correct pL1 plasmids
pL1/GGS	4/20
pL1/Cyclase	1/20
pL1/nptII	1/10
pL1/P450-1	1/10
pL1/P450-2	2/20
pL1/P450-3	3/10
pL1/ATF	3/10
pL1/SDR	1/10

4.3.6 Module generation: making level 2 plasmids

Numerous attempts were made to directly construct the complete level 2 plasmid containing all the pleuromutilin pathway genes, along with nptII for transformant selection *in planta*, however despite repeated attempts, no correctly constructed plasmids were obtained. Therefore, it was decided to initially work with only the early-stage genes to make plasmid construction easier and to validate these before moving to full pathway expression. GGS and Cyclase are the key enzymes responsible for the synthesis of 3-deoxo-11-dehydroxy-mutilin, the first metabolite in the pleuromutilin biosynthesis pathway, so the initial step of this project was to construct an *Agrobacterium* plasmid containing GGS and Cyclase using the Golden Gate assembly system. The GGS/Cyclase plasmid could then be assessed via a transient expression in tobacco leaves, to assess the production of the first metabolite in pleuromutilin pathway *in planta*, this validation would help support the plan to then express the entire pathway.

A *Bpi*I-based Golden Gate cloning reaction was set up, including all three level 1 modules (pL1- GGS, pL1-Cyclase and pL1-nptII), a matching end-linker (EL), and a level 2 destination vector, creating the final expression vector pL2- GGS/Cyclase/nptII (Figure 40). All level 2 destination vectors confer resistance to kanamycin and encode a red colour counter selectable marker, SRed, for a red/white selection for a correctly assembled level 2 construct.

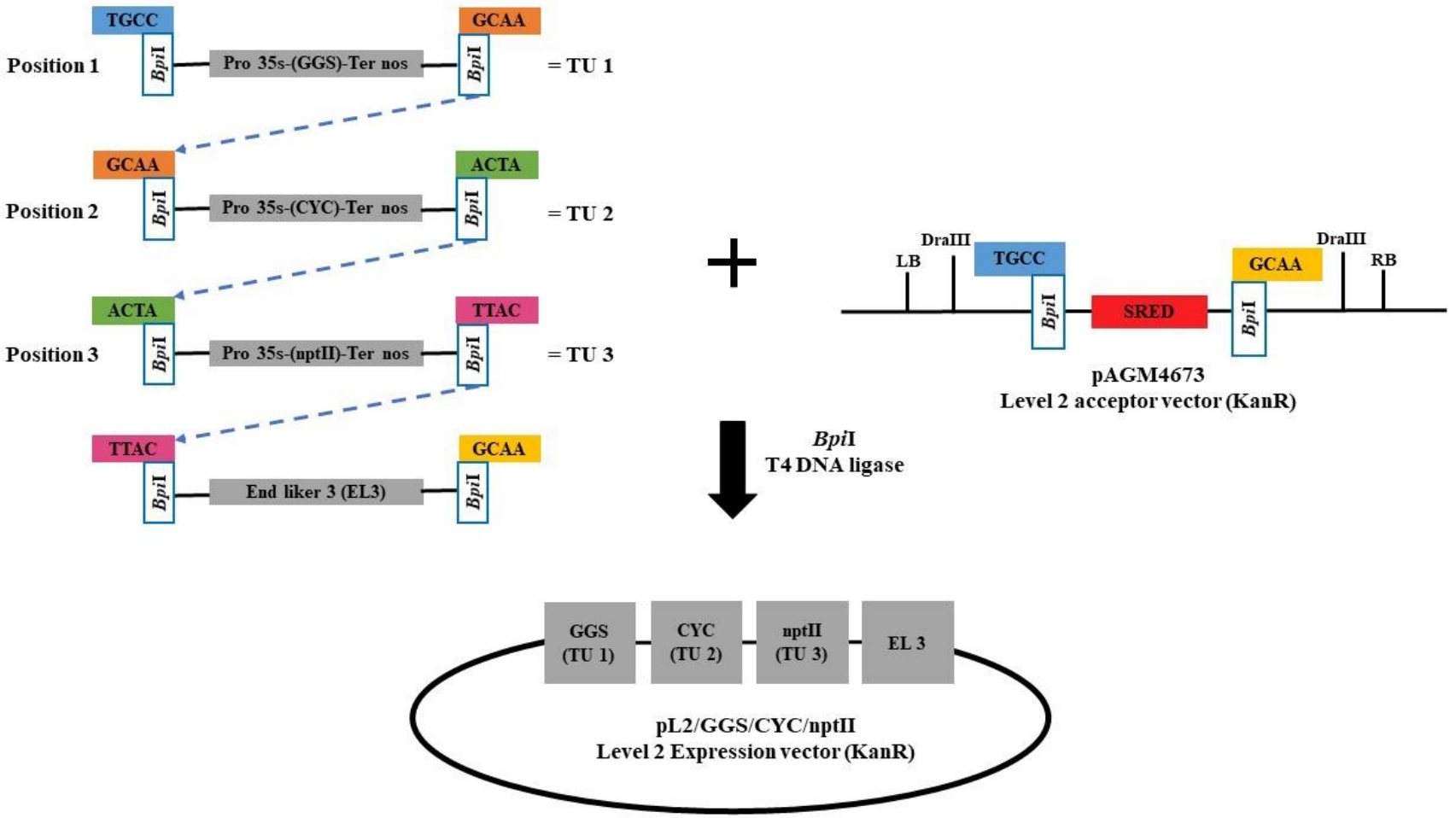


Figure 40 A method for the construction of the level 2 expression vector containing GGS and Cyclase. The compatible set of transcription units for each gene was assembled together by the Golden Gate assembly reaction via *Bpi*I.

The level 2 Golden Gate reaction for construction of pL2- GGS/Cyclase/nptII plasmid contained: 100 ng pAGM4673 plasmid (pL2 acceptor vector), 200 ng pL1-GGS, 200 ng pL1-Cyclase, 200 ng pL1-nptII, 200 ng pEL-3, 1 µL *Bpi*I, 1 µl T4 DNA ligase enzyme, 2 µl T4 DNA ligase buffer, 2 µl Buffer G and was adjusted with water to 20 µl. The mixture was transformed into competent *E. coli* cells and plated on LB plates containing kanamycin. All ten transformant colonies tested showed the successful assembly of GGS, Cyclase and nptII genes when assessed by PCR (Figure 41). One plasmid was further confirmed by restriction digest with the appropriate enzyme (Figure 42).

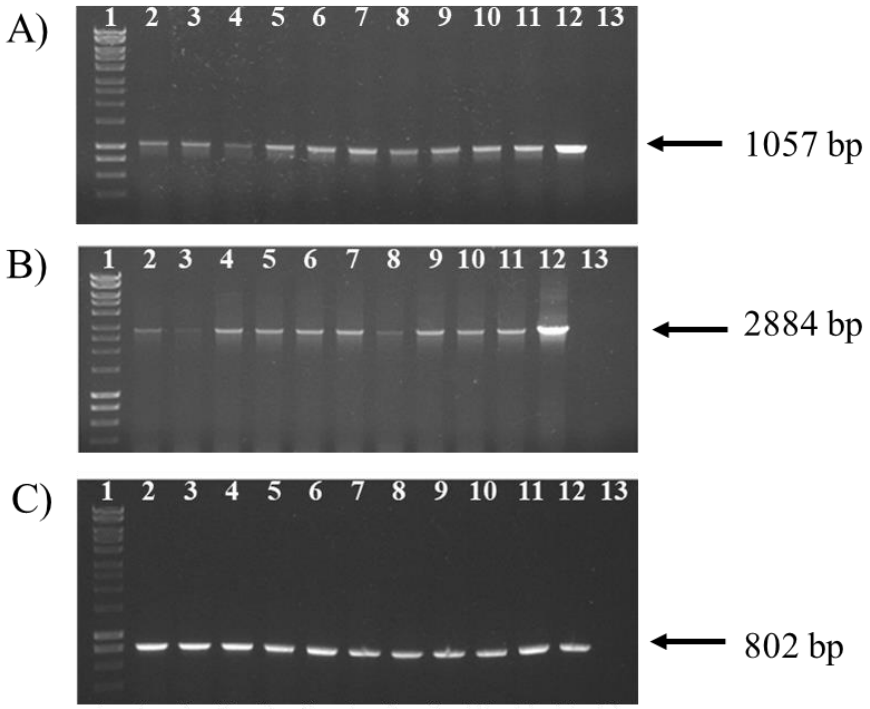


Figure 41 PCR amplification products of GGS, Cyclase and nptII genes from pL2-GGS/Cyclase/nptII expression vector. Ten colonies of *E. coli* transformants were screen for the genes inserted: A) GGS (expected fragments 1057 bp), B) Cyclase (expected fragments 2884 bp) and C) nptII (expected fragments 802 bp). Lanes from left to right: 1 = 5 µL of hyperladder I (Bioline), 2-11 = ten colonies of *E. coli* transformants, 12 = the plasmids pYES2/mGGS, pYES2/mCyclase and pCAMBIA2300 were used as positive controls and 13 = water was used as a negative control.

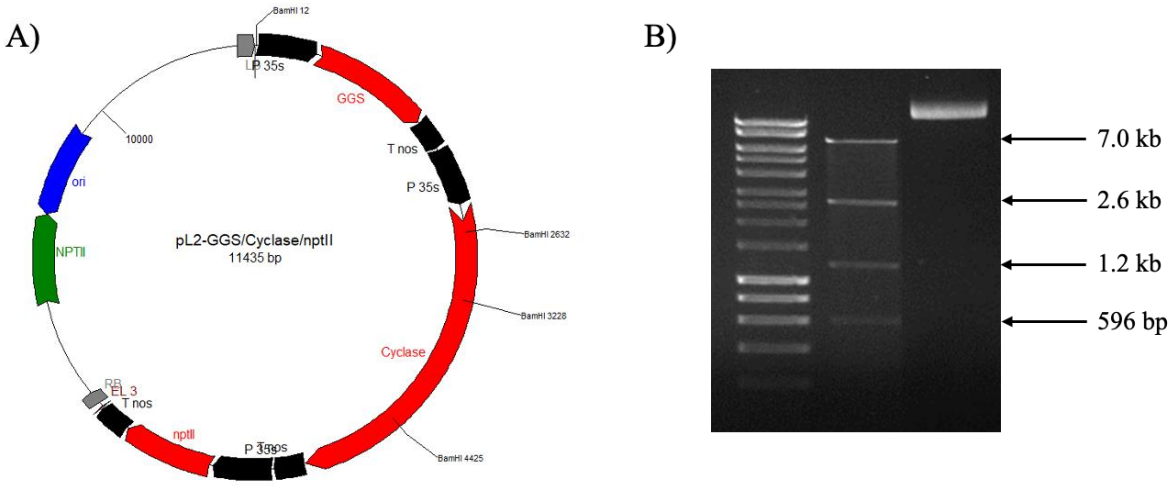


Figure 42 A) Plasmid map of pL2- GGS/Cyclase/nptII and B) Restriction digest showing the correct construction: lane 1 contains 5 µL of hyperladder I (Bioline), lane 2 contains 10 µL of digestion reaction with restriction enzyme *BamH*I of pL2- GGS/Cyclase/nptII (expected fragments 7.0 kb, 2.6 kb, 1.2 kb and 596 bp) and lane 3 contains 1 µL of undigested plasmid DNA of pL2- GGS/Cyclase/nptII.

4.3.6.1 Assessing transient expression of pL2- GGS/Cyclase/nptII in *N. tabacum*

The GGS/Cyclase expression plasmid was transformed into electro-competent cells of *A. tumefaciens* strain LBA4404 with selection for kanamycin cassette. Liquid culture was prepared and induced with acetosyringone to infiltrate tobacco leaves to detect products by transient expression. Three leaves of each plant were infiltrated 4-5 times with 1 mL of culture and plants maintained in a growth cabinet for three days. After three days, the infected leaves were harvested and extracted with 100 mL ethyl acetate.

The GGS and Cyclase genes was expected to produce, 3-deoxo-11-dehydroxy-mutilin (metabolite 1), the first metabolite in pleuromutilin biosynthesis pathway and previous research by Alberti et al., (2017) showed that this metabolite would be able to detect by TLC.

The crude extract (2 µL) of *N. tabacum* pL2- GGS/Cyclase/nptII was separated on TLC and compared with the extract from *A. oryzae* GC-pdc TS2-2 (used as positive control) and *N. tabacum* containing empty vector (used as negative control) (Figure 43). The results had expected a high mobility product as previously seen in the Chapter 3. Unfortunately, the infiltrated tobacco gave a very complex profile on the TLC which prevented detection of 3-deoxo-11-dehydroxy-mutilin (metabolite 1).

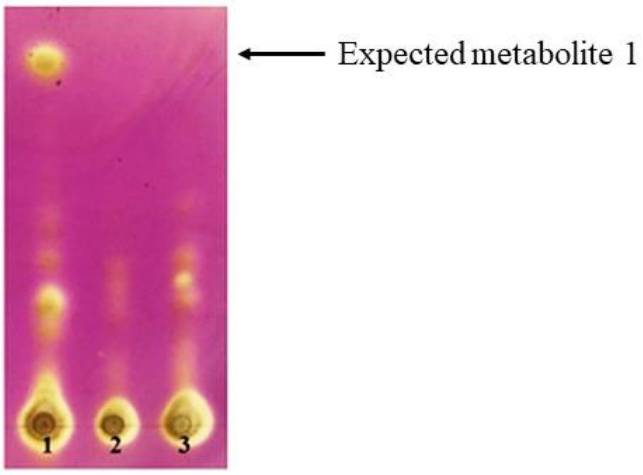


Figure 43 Thin layer chromatography analysis of the crude extract from *N. tabacum* pL2-GGS/Cyclase/nptII compared with *A. oryzae* GC-pdc TS2-2. Spot 1: crude extract of *A. oryzae* transformant strains TS 2-2 containing GGS and Cyclase, spot 2: crude extract of *N. tabacum* containing pL2 containing empty vector and spot 3: crude extract of *N. tabacum* harbouring expression vector pL2- GGS/Cyclase/nptII.

4.3.7 Module generation: the level 2 of the final expression vector for the GGS, Cyclase and P450-1 genes

Given the unsuccessful detection of 3-deoxo-11-dehydroxy-mutilin from the transient *in planta* assay, construction of the full expression plasmid was restarted. The pL2-GGS/Cyclase/nptII/P450-1 was constructed to add the P450-1 gene, which expected to generate 3-deoxo mutilin (metabolite 2). This metabolite would be more polar compared to metabolite 1 and would be able to be analysed through HPLC.

The Golden Gate assembly to make the level 2 plasmid pL2- GGS/Cyclase/nptII/P450-1 was undertaken through the reaction mixture containing: 100 ng pAGM4673, 200 ng pL1-GGS, 200 ng pL1-Cyclase, 200 ng pL1-nptII, 200 ng pL1-P450-1, 200 ng pEL-4, 1 µL BpiI, 1 µl T4 DNA ligase enzyme, 2 µl T4 DNA ligase buffer, 2 µl Buffer G and adjusted with water to 20 µl. Then the mixture was transformed into competent *E. coli* cells and plated on LB plates containing kanamycin. Successful construction of pL2-GGS/Cyclase/nptII/P450-1 was confirmed with PCR amplification of each of the genes inserted and restriction digest with the appropriate enzyme (see Figure 44).

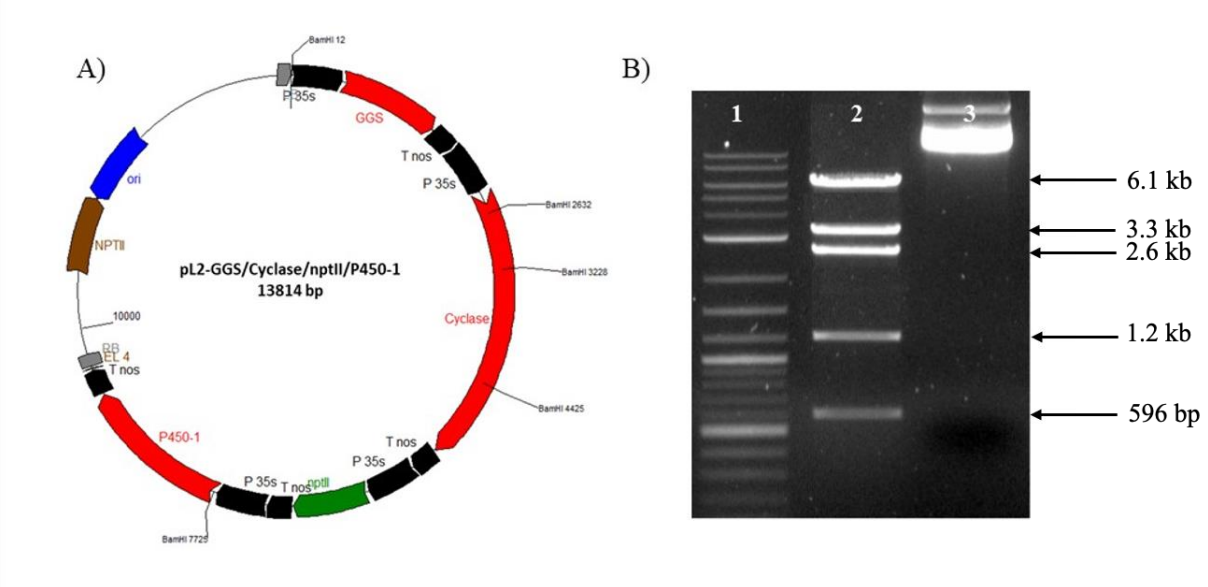


Figure 44 A) A map of predicted pL2- GGS/Cyclase/nptII/P450-1. B) Restriction digest showing correct construction of plasmid pL2- GGS/Cyclase/nptII/P450-1: lane 1 contains 5 µL of 2-log ladder, lane 2 contains 10 µL of digestion reaction with restriction enzyme *BamH*I of pL2- GGS/Cyclase/nptII (expected fragments 6.1 kb, 3.3 kb, 2.6 kb, 1.2 kb and 596 bp) and lane 3 contains 1 µL of undigested plasmid DNA of pL2-GGS/Cyclase/nptII/P450-1.

4.3.7.1 Chemical analysis of transgenic *N. tabacum* with pL2- GGS/Cyclase/nptII/P450-1

The successful pL2-GGS/Cyclase/nptII/P450-1 construct was used to transform electro-competent cells of *A. tumefaciens* strain LBA4404. Liquid culture inducted with acetosyringone were used to infiltrate tobacco leaves to induce transient expression. Five 4-6-week-old plants of *N. tabacum* were used for *Agrobacterium* infiltration. Three leaves of each plant were infiltrated 4-5 times with 1 mL of culture and plants maintained in a growth cabinet for three days. After 3 days, the infected leaves were harvested and extracted with 100 mL ethyl acetate. The crude extract of *N. tabacum* pL2- GGS/Cyclase/nptII/P450-1 was detected through preparative HPLC compared with *N. tabacum* containing pL2 empty vector (used as negative control).

From the ELSD chromatogram, no peak was eluted having the same m/z with expected metabolite 2. The mass of expected metabolite 2 was calculated to be = 351 m/z following the calculation previously used by Alberti et al., (2017). The predicted m/z value of metabolite 2 was scanned through the MS spectrum using MassLynx software but the attempt failed to find any trace from the extract in the negative mode (ES^-) as shown in Figure 45.

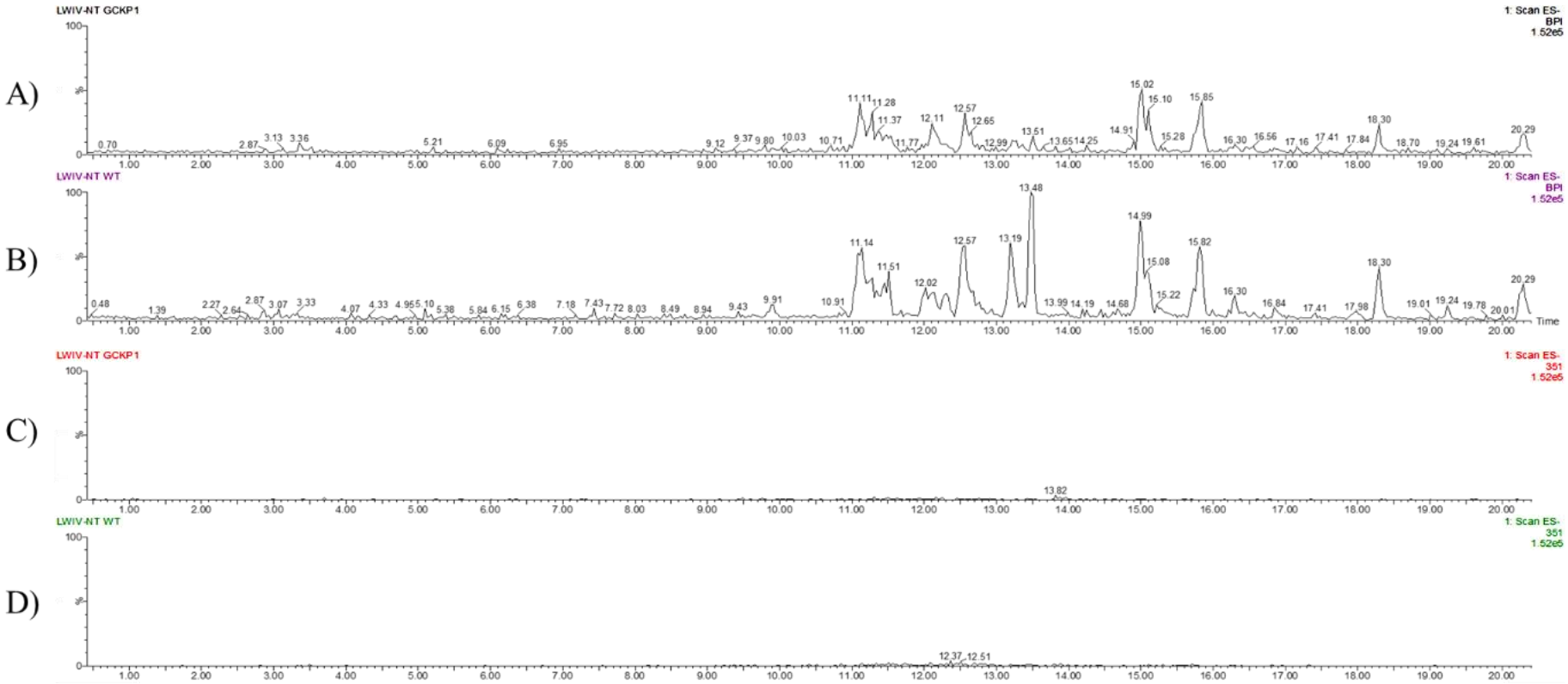


Figure 45 Attempted detection of metabolite 2 from infiltrated tobacco. A and B) LCMS (ES^-) spectrum from infiltrated and non-infiltrated leaves. C and D) Extracted ion traces (ES^- -351) spectrum from infiltrated and non-infiltrated plants. This shows no readily detectable metabolite with m/z 351.

4.3.8 Module generation: the level 2-1 expression vector for the GGS, Cyclase, P450-1, P450-2 and P450-3 genes (5 genes)

Further attempts to create the seven genes of pleuromutilin biosynthesis pathway assembled into level 2 construct were designed. Due to a large size plasmid construction and as the maximum number of transcription unit was limited to six which could be cloned in one round into a level 2 acceptor vector, the final construction was divided into two rounds: L2-1 and L2-2.

The Golden Gate assembly of level 2 for pL2-1: GGS/Cyclase/nptII/P450-1/P450-2/P450-3 plasmid was undertaken through the reaction mixture containing: 100 ng pAGM4673 plasmid, 200 ng pL1-GGS, 200 ng pL1-Cyclase, 200 ng pL1-nptII, 200 ng pL1-P450-1, 200 ng pL1-P450-2, 200 ng pL1-P450-3, 200 ng pEL-6 (lacZ), 1 µL *Bpi*I, 1 µl T4 DNA ligase enzyme, 2 µl T4 DNA ligase buffer, 2 µl Buffer G and adjusted with water to 20 µl. The reaction was run in a thermocycler using the program as described earlier in Chapter 2 Materials and Methods section 2.2.14. Then the mixture was transformed into competent *E. coli* cells and plated on LB plates containing kanamycin. Five transformant colonies showed the successful assembly of pL2-1 when assessed by PCR amplification of the genes inserted and restriction digest with the appropriate enzyme (see Figure 46).

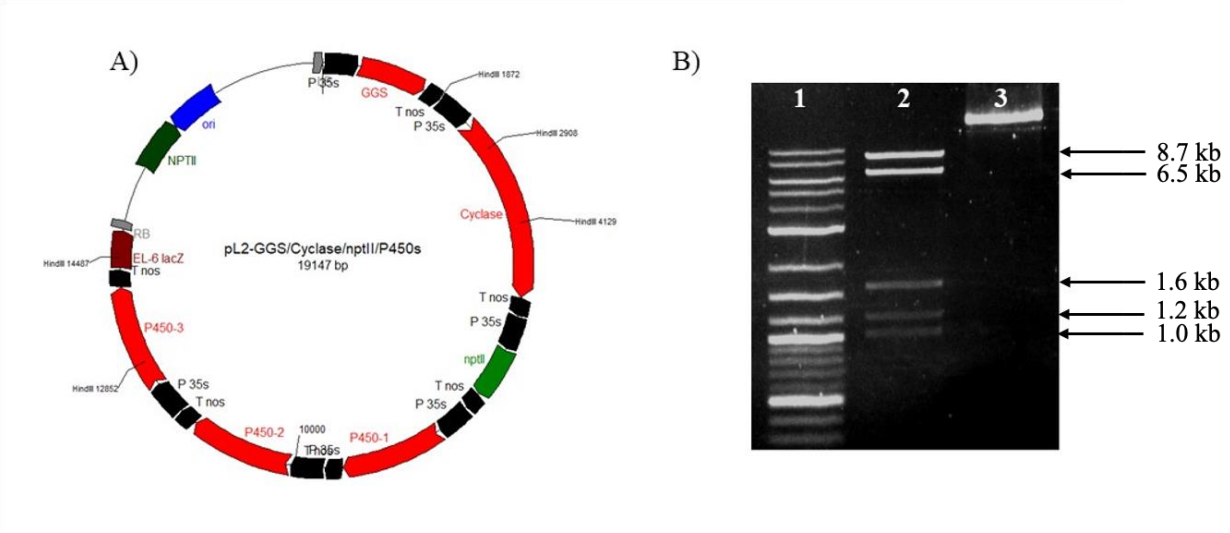


Figure 46 A) Predicted map for pL2- GGS/Cyclase/nptII/P450s B) Restriction digest showing correct construction of plasmid pL2-1 GGS/Cyclase/nptII/P450s: lane 1 contains 5 µL of 2-log ladder, lane 2 contains 10 µL of digestion reaction with restriction enzyme *Hind*III of pL2- GGS/Cyclase/nptII/P450s (expected fragments 8.7 kb, 6.5 kb, 1.6 kb, 1.2 kb and 1.0 kb) and lane 3 contains 1 µL of undigested plasmid DNA of pL2-GGS/Cyclase/nptII/P450s.

The correctly assembled L2-1 was then used for the final round of L2-2 plasmid construction for the complete seven genes of pleuromutilin pathway assembly.

4.3.9 Module generation: making the level 2-2 final expression vector for the seven genes of the pleuromutilin biosynthetic pathway

The final round assembly for level 2-2 contains all seven genes involved in pleuromutilin biosynthesis pathway. The successful construct of pL2-1 was used as the destination vector for the addition of the last two transcription units: pL1-ATF and pL1-SDR.

The level 2 Golden Gate assembly for the pL2-2 seven genes plasmid was undertaken through the reaction mixture containing: 100 ng pL2-1 plasmid, 200 ng pL1-ATF, 200 ng pL1-SDR, 200 ng pEL-1, 1 µL *Bpi*I, 1 µL *Eco*31I, 1 µl T4 DNA ligase enzyme, 2 µl T4 DNA ligase buffer, 2 µl Buffer G and adjusted with water to 20 µl. The reaction was run in a thermocycler using the program as described previously (section 2.2.14). The mixture was transformed into competent *E. coli* cells and plated on LB plates supplemented with kanamycin and X-gal. Three plasmids were confirmed by PCR amplification for the presence of all genes and restriction digest with the appropriate enzyme (Figure 47 and 48). Positive results were observed from all three plasmids

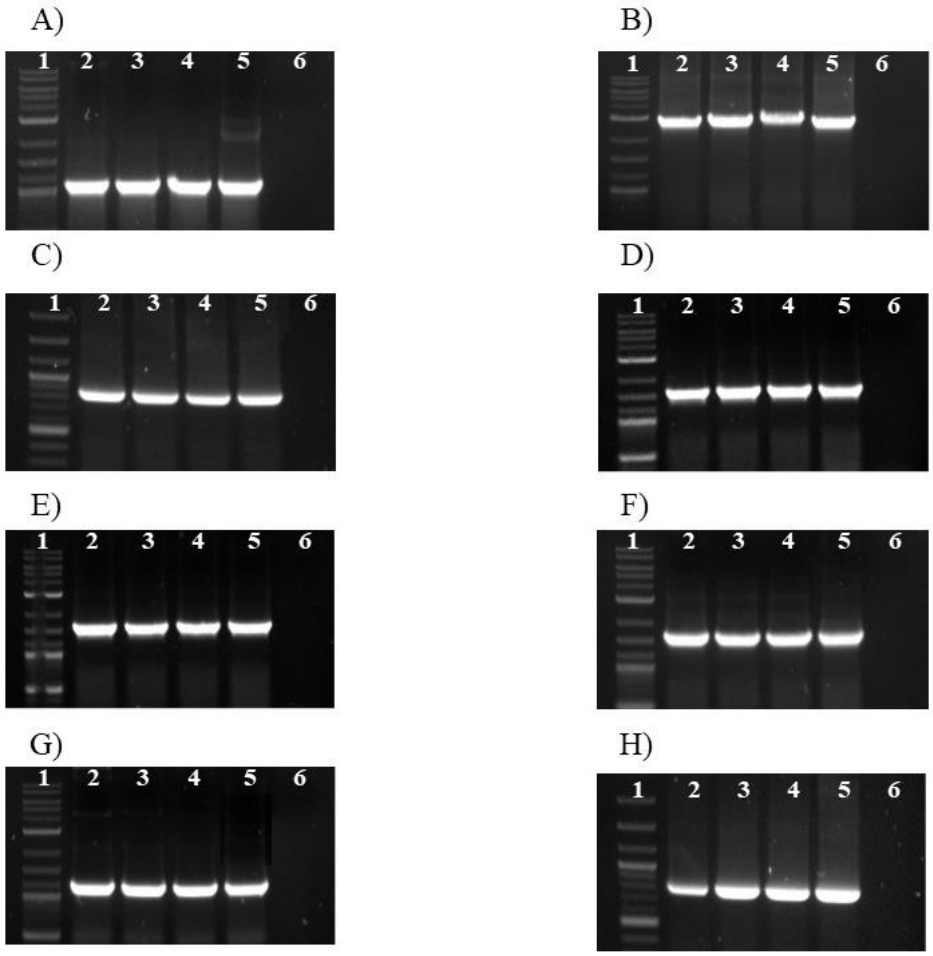


Figure 47 PCR analysis of the level 2-7 plasmid confirming amplification products for GGS, Cyclase, nptII, P450-1, P450-2, P450-3, ATF and SDR genes.

Lane 1 contains 5 μL of 2-log ladder: lanes 2-4 the extracted plasmids pL2-7 were used as a template; A) PCR amplification of the GGS gene (expected fragments 1057 bp), B) PCR amplification of the Cyclase gene (expected fragments 2884 bp), C) PCR amplification of the nptII gene (expected fragments 798 bp), D) PCR amplification of the P450-1 gene (expected fragments 1576 bp), E) PCR amplification of the P450-2 gene (expected fragments 1582 bp), F) PCR amplification of the P450-3 gene (expected fragments 1573 bp), G) PCR amplification of the ATF gene (expected fragments 1138 bp), H) PCR amplification of the SDR gene (expected fragments 766 bp), in lane 5 the plasmid pL0 of each gene was used as a positive control and lane 6 water was used as a negative control.

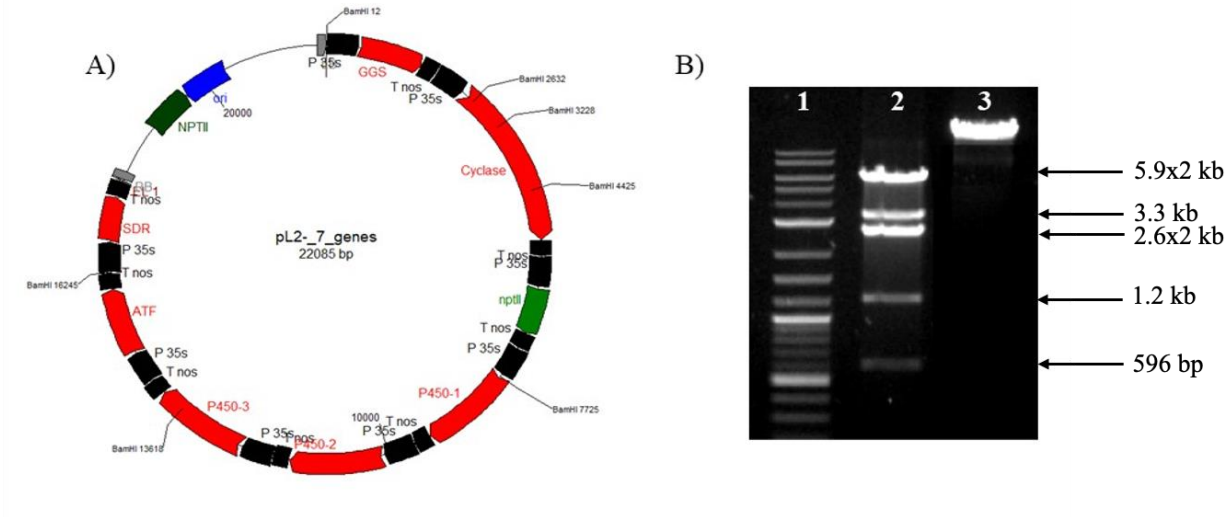


Figure 48 A) Map of the 7-gene plasmid pL2-7; GGS/Cyclase/nptII/P450-1/P450-2/P450-3/ATF/SDR. B) Restriction digest showing correct construction of plasmid pL2-7: lane 1 contains 5 µL of 2-log ladder, lane 2 contains 10 µL of digestion reaction with restriction enzyme BamHI of pL2-7 (expected fragments 5.9x2 kb, 3.3 kb, 2.6x2 kb, 1.2 kb, and 596 bp) and lane 3 contains 1 µL of undigested plasmid DNA of pL2-7.

4.3.10 *Agrobacterium* infiltration of *N. tabacum*

N. tabacum was chosen as a host to assess the transient expression of genes involved in pleuromutilin biosynthesis pathway. Four expression vectors; 1) pL2/GGS/CYC/nptII, 2) pL2/GGS/CYC/nptII/P450-1, 3) pL2/GGS/CYC/nptII/P450-1/P450-2/P450-3 and 4) pL2-7 were used for *Agrobacterium* infiltration (Figure 49). Each plasmid was transformed into electro-competent cells of *A. tumefaciens* strain LBA4404 and was used to infiltrate tobacco leaves. Phenotype evaluation was observed and photographed every two days.

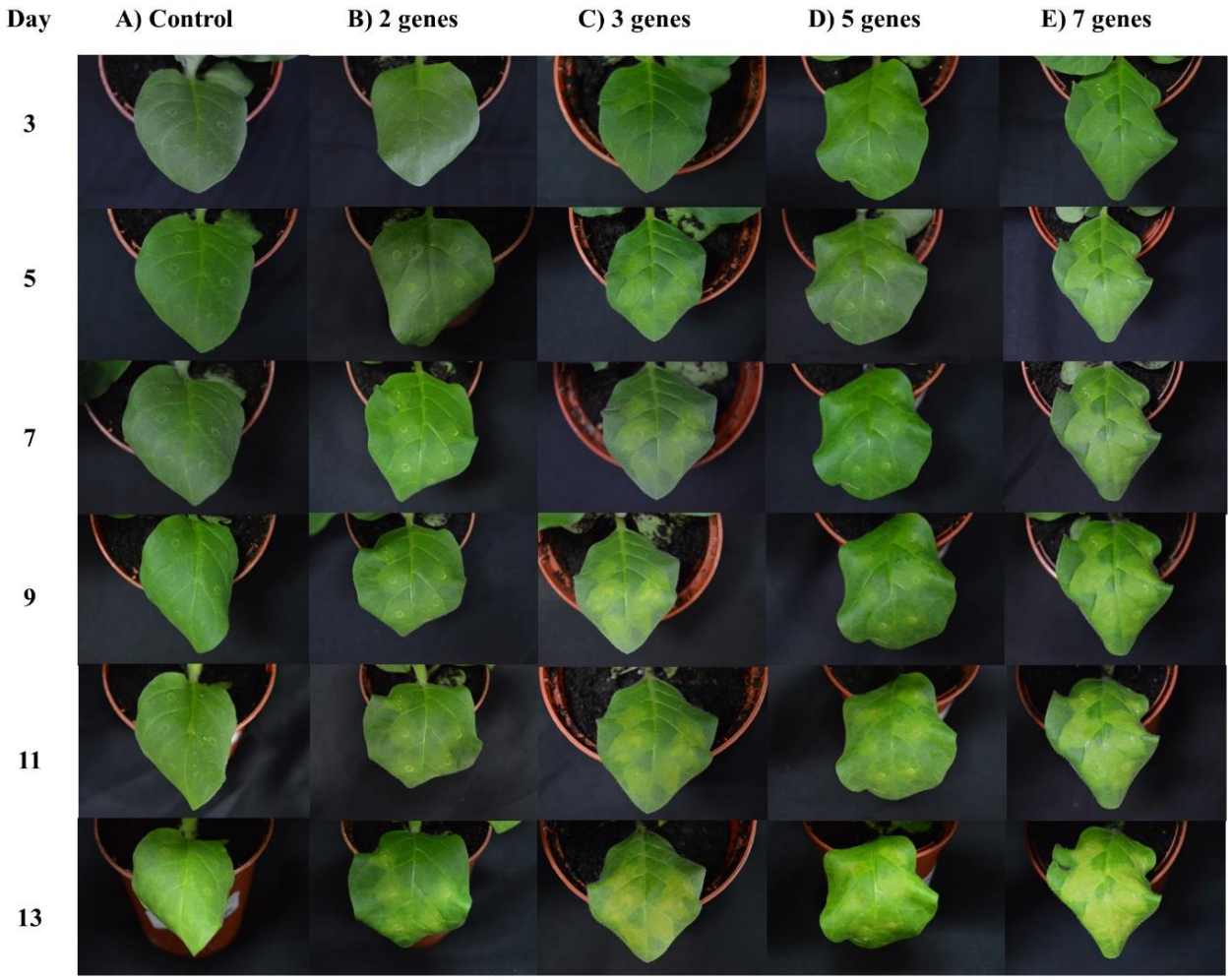


Figure 49 The progressive development of symptoms in *N. tabacum* every 2 days after infiltrating with *Agrobacterium* containing pL2 expression vector harbouring various genes involved in the pleuromutilin biosynthesis pathway; A) Control = empty pL2 vector, B) 2 genes = *GGS* and *Cyclase*, C) 3 genes = *GGS*, *Cyclase* and *P450-1*, D) 5 genes = *GGS*, *Cyclase*, *P450-1*, *P450-2* and *P450-3* and E) seven genes of pleuromutilin cluster.

After 13 days of observation, the leaves which were infiltrated with each plasmid presented the yellowish lesions from the infiltration spots, while no lesion was found on the empty vector. Among them the leaf infiltrated with the 7 genes plasmid showed more extensive chlorosis when compared with other infiltrated leaves (Figure 49E). In addition, the development of chlorosis seemed to increase correlated with the number of genes in the expression vector. Alberti et al., (2017) showed increasing antibacterial properties for mutilin compounds as they progress towards being the final mature product and the observed increase in chlorosis seems to correlate with this.

Results suggest constructs are active, but the resulting product is below the threshold for detection with this method or is not extractable from the leaf. The chlorosis suggests *in planta* production is toxic so a stable transgenic route is not appropriate with these vectors, as transformant callus tissue would likely be non-viable or fail to generate.

4.4 Discussion

Plant-based systems are now gaining acceptance as reliable expression platforms for heterologous expression, especially for the production of recombinant proteins in the pharmaceutical industry. Among various plant species, tobacco is one of the most suitable hosts to be used as a heterologous system for the production of secondary metabolites. This plant-based system provides numerous advantages over the other plants; as a leafy plant it is capable of the production of high biomass, it has well-established methods for transformation procedures, it is classified as a non-food, non-feed crop so it reduces risk of contamination in the feed or food chain (Abiri et al., 2015, Fischer et al., 2004). To date, various types of vaccines and antibody have been produced in tobacco. A full length of a mouse catalytic IgG1(6D4) monoclonal antibody was the first antibody generated in tobacco (Hiatt and Pauly, 2006), as well as other antibodies that targeted different diseases such as anthrax, hepatitis, rabies etc. (Girard et al., 2006, Gleba et al., 2005, Hull et al., 2005). So, tobacco was chosen as a new platform for heterologous expression of pleuromutilin biosynthesis pathway within this project.

The selection of an appropriate plant expression vector was another crucial factor for this project. The goal is to recreate the whole pleuromutilin biosynthesis pathway then transfer for heterologous expression *in planta*. However, the pathway consists of seven genes all of which need refactoring for expression. In the fungal host *A. oryzae*, the seven genes were divided into three plasmids and several rounds of transformations were performed. A similar procedure could be employed in tobacco, but it was considered as a time-consuming process. In order to simplify the multi genes assembly, the Golden Gate assembly method was chosen as a strategy to assemble the seven genes of pleuromutilin pathway into plant expression vector for this project. The method based on the ability of type IIS enzymes was proven to be efficient and reliable for the multiple fragments assembly (Engler et al., 2008, Engler et al., 2014, Lampropoulos et al., 2013, Weber et al., 2011) and the availability of well-validated promoter and terminator regions in the toolkit.

At the beginning numerous attempts were performed to assembly the seven genes involved in the pleuromutilin production, but after countless repeated trials no correct construct could be obtained suggesting the process was not as simple as the literature had claimed. Therefore, the construction of the expression vector containing just the GGS and Cyclase genes involved in pleuromutilin biosynthesis pathway was done by using the Golden Gate cloning reaction.

The effort was to focus on the bottleneck step to enhance the production of the most upstream precursor to go through the other steps along the pathway and result in the increase of pleuromutilin titre at the end. Results from TLC analysis of infiltrated *N. tabacum* pL2-GGS/Cyclase/nptII did not show a spot of the expected metabolite 1, but the likely reason might due to numerous compounds produced *in planta* which could be interfered spot of the expected metabolite, the production was not confirmed.

Next step approach was to add P450-1 gene into the expression vector with the aim of generating the metabolite 2 which has a higher polarity and would be able to be investigated it through preparative HPLC. Unexpectedly, chemical analysis failed to detect any trace of metabolite 2 by HPLC. There are several possible reasons to account for the lack of accumulation of the expected metabolite in tobacco. The small batch of plant samples with just five plants might lead to a low yield of metabolite 2 in the crude extract or the fungal P450 may not function in a plant cell.

Another issue that has to be considered is the production of unexpected compound resulting from the modification by endogenous enzymes of the host system. Various reaction such as oxidation, glycosylation and dephosphorylation within the host system might give chance to produce a new compound, but it is also the possibility of having produced a toxic compound and since plants evolved many defensive mechanisms against pathogens and environmental stress (Reed and Osbourn, 2018, Zhang et al., 2011), any possible metabolite produced by these three genes might be detected and broken down by the host before it can be accumulated.

Many attempts failed to construct the seven genes plasmid at the beginning of the project as the construction did not work according to the theoretically principle. With Golden Gate working more reliable it was feasible to make a wide range of vectors, but this highlights the need for experience work for the system to be able to use it efficiently. The key point to be noted is the number of cycles for the restriction-ligation reaction. The results from this project correlated to the results from prior studies (Engler et al., 2008). that when comparing the short reaction of 30 cycles and the long reaction of 50 cycles, the long cycle showed a significant increase in the number of the desired colonies. In addition, many false positive colonies were found within this project. The sequence analysis of those constructs was not done in this project. In the other studies, it was suspected that the possible reason for this

might be the formation of dimer structures of the vectors and inserts (vector-insert-vector-insert) of the recombinant plasmids but this was not performed in this study.

Despite many difficulties, the final attempt was successful to construct the expression plasmid containing all seven genes of pleuromutilin biosynthesis pathway. The investigation of transient gene expression of the seven genes in tobacco was carried out, the result showed a yellowish lesion on transgenic plants but none on the empty vector construct. Even though it was not clear that pleuromutilin metabolites were produces *in planta*, it could be assumed that there was some production of an unknown metabolite in the plants that was associated with these genes products, however, the production of the unknown substance seemed to be toxic to the plants. Many possibilities could exist for the occurrence of the yellowish lesion as follows.

Firstly, if the initial hypothesis was right, the seven genes in pleuromutilin cluster was expressed and resulted in the production of pleuromutilin *in planta*. These pleuromutilin metabolites may affect the growth or survival of the plant. Pleuromutilin antibacterial activity is by binding to the ribosome of bacteria and inhibiting its protein synthesis. The biological evolution points out that the organelles such as mitochondria or chloroplast in eukaryote were of bacterial origin. According to the endosymbiosis theory, the organelles in eukaryote cells could be originated from endophytic prokaryote organisms when the eukaryote cell engulfed the prokaryote through symbiosis. Therefore, the organelle ribosomes in plants could be similar to the ones in the bacteria and hence sensitive to pleuromutilin (Gruber, 2019, Zimorski et al., 2014). This is probably the best explanation why the plant is affected by pleuromutilin. It was not investigated whether pleuromutilin is toxic to the chloroplast or mitochondrial ribosome, but the extensive chlorosis might indicate a chloroplast-based effect.

In nature, plants produce and store diterpene compounds in leucoplasts (Zi et al., 2014). Leucoplasts differ from chloroplasts because they lack photosynthetic pigments. The biosynthetic genes for the production of monoterpene limonene have been found to be highly expressed in glandular trichomes (Jongedijk et al., 2016). A similar result was also found in the study of the biosynthesis of the well-known sesquiterpene compound, artemisinin, showing that it is produced in the cells of trichromes (Zhang et al., 2011). Therefore, it should be considered that the specific cell type in plants may provide the cellular compartment and biochemical environment that is suitable for the production of terpene compounds and it is unclear whether the transient *Agrobacterium* infiltration would deliver the gene to these cells.

In addition, the 35S promoter of the cauliflower mosaic virus was used for the constitutive expression of the genes in this study. It would be interesting to investigate the use of tissue specific promoters or inducible promoters to drive the genes. The inducible promoters may provide a possibility to induce or upregulate the genes at a specific stage of plant development or specific tissue of interest, then allow the metabolites to accumulate (Borghi, 2010). A previous study reported success using an ethanol-inducible promoter to enhance the levels of recombinant protein expression upon induction, up to 4.3 mg/g fresh biomass in transgenic *Nicotiana benthamiana* plants (Werner et al., 2011). Such an approach may work for increasing metabolite levels.

A different possibility is that the heterologous expression of P450 genes did not function correctly in the plant host system. The activity of P450s are dependent on the supply of NAD(P)H and electrons. Cytochrome P450 reductase, as a redox partner of the P450 monooxygenase, also plays a crucial role to achieve optimal P450s activity (Durairaj et al., 2016). The work from Zhang et al., (2011) showed the possibility of the accumulation of artemisinin precursors through the heterologous expression of *A. annua* cytochrome P450 (CYP71AV1) and artemisinic aldehyde Δ11(13) double-bond reductase (DBR2) in transgenic tobacco. Even though, the result of their study might give hope for the heterologous expression of P450 gene in plant system, but this was a heterologous expression of plant P450 in another plant. The expression of fungal P450s *in planta* has not been reported in any previous study yet, so in this study plant Cytochrome P450 reductase might not reactivate fungal P450s. To my knowledge, this project might be the first attempt for the heterologous expression of fungal genes *in planta*.

As the expression analysis of the genes was not carried out within the project, it is unknown whether the genes were expressed or not, but the promoters are all well-validated and so likely to be functional. Further investigation is needed to be carry out for the understanding of the mechanisms of the enzymes and to ensure that the full potential of enzyme activities could lead to the production of the desired compound.

4.5 Summary

The work in this chapter provided the information for the alternative platform for the heterologous expression of genes encoding the pleuromutilin biosynthesis pathway in infiltrated tobacco. The results failed to detect the desired intermediate through HPLC analysis. This could indicate many possibilities; the yield was too low, the cellular environment *in planta* was not suitable for the metabolite production or it was broken down by the host defensive mechanisms before it could be accumulated within the plant. Nevertheless, the expression of fungal genes in plant remain unexplored and unexploited. The further challenge and a better understanding of the fungal genes in plant could open the possibilities to generate a reliable platform for fungal metabolite production for various applications in the future.

Chapter 5 Heterologous expression of pleuromutilin biosynthetic genes in yeast system

5.1 Introduction

The heterologous expression of the pleuromutilin biosynthetic pathway in the fungal host system, *A. oryzae* and plant-based system, *N. tabacum* did not give the expected result in the improvement of pleuromutilin production. Therefore, the yeast *Saccharomyces cerevisiae*, another promising heterologous host system, was chosen as an alternative host for the expression of this antibiotic gene cluster.

Terpenoids are the largest class of natural products, which include more than 50,000 compounds that have significant commercial applications ranging from food flavours, fragrances, cosmetics to medicines (Ignea et al., 2011). Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the two general precursors for the biosynthesis of all terpenoid compounds. The IPP and DMAPP units are derived through either the mevalonate pathway (MVA) or the methylerythritol phosphate pathway (MEP) (Zhang et al., 2017). In yeast IPP is synthesized through the MVA pathway. The condensation of IPP and DMAPP, catalytic reaction through *ERG20* gives rise to higher building blocks of geranyl pyrophosphate (GPP; C_{10}), farnesyl pyrophosphate (FPP; C15) and geranylgeranyl pyrophosphate (GGPP; C_{20}), which are the substrates of mono-, sesqui- and di- terpene synthases, respectively. The action of those terpene synthases together with their substrate will generate the terpene hydrocarbon scaffold of monoterpenes, sesquiterpenes and diterpenes (Figure 50). Yeast does not normally produce many terpenoids as most of the product from the MVA pathway are used for sterol biosynthesis. However, previous studies in yeast showed the successful expression of a plant terpene synthase derived from *T. chinensis* for the production of taxol, a diterpene compound used as an anticancer agent and another plant terpene synthase derived from *A. annua* for the production of artemisinin, a sesquiterpene compound used against malaria. This demonstrated that it was possible for the exogenous terpene synthase enzymes to use the endogenous substrates for the production of various terpenoid compounds (Kampranis and Makris, 2012).

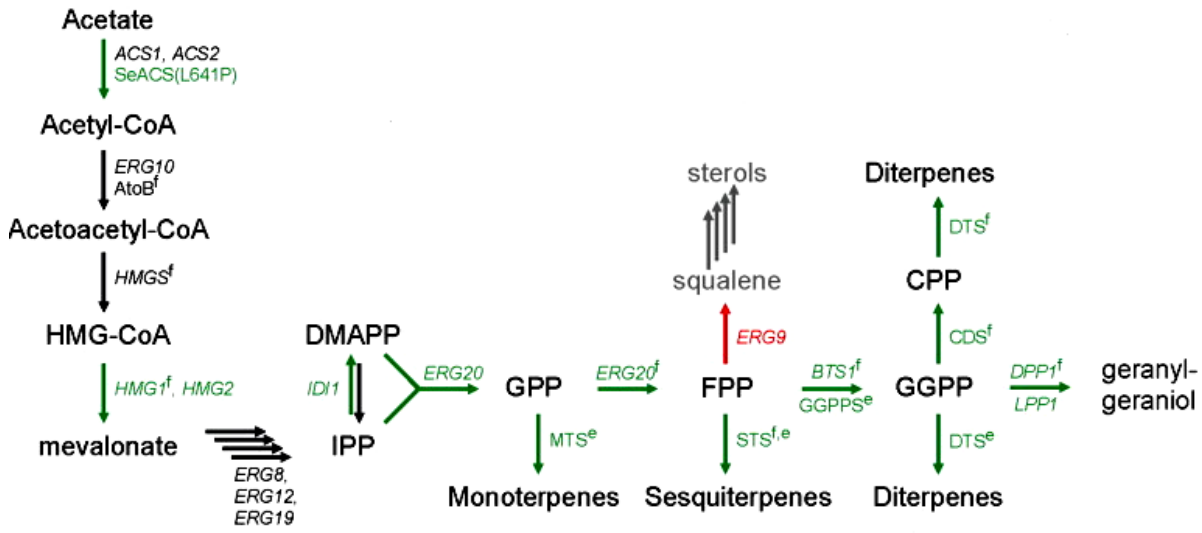


Figure 50 Illustrated the mevalonate pathway for terpenoids biosynthesis in yeast. Modified from Kampranis and Makris, (2012).

5.1.1 Successful metabolic engineering cases for the production of terpenoids in yeast

5.1.1.1 Taxol

The recent metabolic engineering study by Engels et al., (2008) demonstrated a successful production of taxadiene, a precursor of taxol in yeast *Saccharomyces cerevisiae*. Taxol is a diterpene compound, originally extracted from the bark of the pacific yew tree (*Taxus brevifolia*). Taxol and its derivatives are known as effective chemotherapy agents use for the treatment of cancer. The obstacle that prevents its large-scale production is the low yield of the extract from its natural plant source. Despite a successful total chemical synthesis (Holton et al., 1994, Nicolaou et al., 1994), its complex structure leads to a laborious and costly processes. The taxol biosynthetic pathway (Figure 51) consists of at least 19 enzyme-catalysed steps starting from the diterpene precursor GGPP, followed by the catalytic reaction via taxadiene synthase to generate the taxa-4(5), 11(12)-diene or also known as taxadiene. Taxadiene then will be oxygenated through several cytochrome P450 monooxygenases to yield taxol (Engels et al., 2008). The metabolic engineering approach to create the taxol biosynthetic pathway in yeast provides the possibility to establish the specific steps within the pathway to focus on the desired compound.

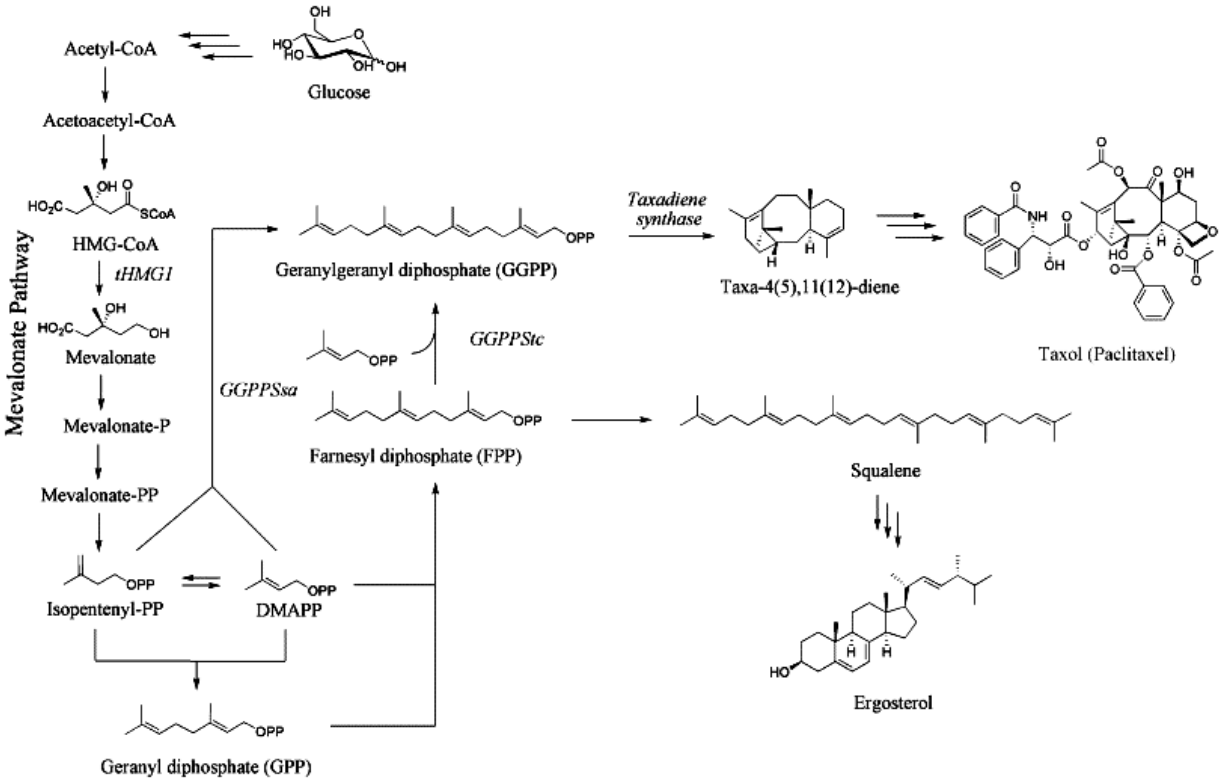


Figure 51 The taxol biosynthetic pathway from Engels et al., (2008).

Engels et al., (2008) reported several approaches to engineer the early part of the taxol biosynthetic pathway for the improvement of yield of the taxadiene compound in the yeast *S. cerevisiae*. Firstly, they introduced the heterologous expression of taxadiene synthase derived from *Taxus chinensis*, but the results showed no accumulation of taxadiene and the possible reason they proposed was an insufficient supply of the precursor GGPP. The next step was the co-expression of taxadiene synthase and GGPP synthase indicate on pathway from *T. chinensis*; the result also failed to show any improvement of taxadiene level. In yeast, most of the output from the MVA pathway is used for the biosynthesis of ergosterol, which is a major component of the cell membrane. The previous study from Ro et al., (2006) showed that by using a shortened version of yeast HMG-CoA reductase, steroid-based negative feedback was prevented and there was increased supply of precursor for other terpene compounds. Using the truncated HMGR, Engels et al., (2008) showed the increase of taxadiene to 306±3 µg/L. Another attempt to inhibit sterol production is based on the mutagenesis of *UPC2*, a key regulatory factor to allow steroid uptake in yeast under aerobic growth condition. The expression of the mutant *UPC2* gene along with the previous three genes, resulted in a 50%

increase of yield of taxadiene. For the last attempt, they replaced the GGPP synthase from *T. chinensis* with the GGPP synthase from a thermophilic archaeon *Sulfolobus acidocaldarius*, in order to increase the GGPP precursor through the DMAPP building block and avoid the competition of steroid synthesis via FPP. The result showed a significant increase of a 40-fold in taxadiene production.

5.1.1.2 Artemisinin

Artemisinin is a sesquiterpene compound with a potent antimalaria property. Its original source is the herb, *Artemisia annua*. Studies showed that artemisinin and its derivatives also have additional pharmacological properties including antiparasite, anticancer, and antiviral activities (Efferth et al., 2008, Keiser and Utzinger, 2007, Wong et al., 2017). A problem with this drug is that the demand and supply are not balanced meaning lots of patients cannot access the drug because of high price (Chandran et al., 2011). Like taxol, the production from its natural source is very limited. The approach for chemical synthesis of the compound is complicated and not cost-effective. The alternative strategies to solve the problem might be by improved agricultural procedures, microbial production, and the development of synthetic processes (White, 2008).

The study from Ro et al., (2006) (Figure 52) reported that engineered *S. cerevisiae* strains could produce a high titre of artemisinic acid. Their first approach started from engineered strains that increase FPP production and decrease it use for sterol synthesis. Use of strain EYP208 overexpressing a short version of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (*tHMGR*), resulted in five-fold increase of amorphadiene production. The strain EYP225 created by downregulation of *ERG9*, the gene responsible for the conversion of FPP into the sterol biosynthetic pathway, resulted in a further increase in titres of amorphadiene production by two-fold. Following that the EPY213 strain was generated with the combination of downregulation of *ERG9* and overexpression of *UPC2-1*, yielded the amorphadiene production up to 105 mg/L. Next *tHMGR* was introduced into the EPY213 strain and generated EPY219, that showed the further increasing of amorphadiene production up to 149 mg/L. Lastly, the final strain EPY224 was engineered by combining all those modifications and including the overexpression of *ERG20*, as a result the production of amorphadiene was up to 153 mg/L. Finally, the EPY224 then was used for the transformation

with vector containing cytochrome P450 monooxygenase together with its redox partner from *A. annua* (CYP71AV1/CPR), the extract from shake-flask culture provided 32±13 mg/L of artemisinic acid.

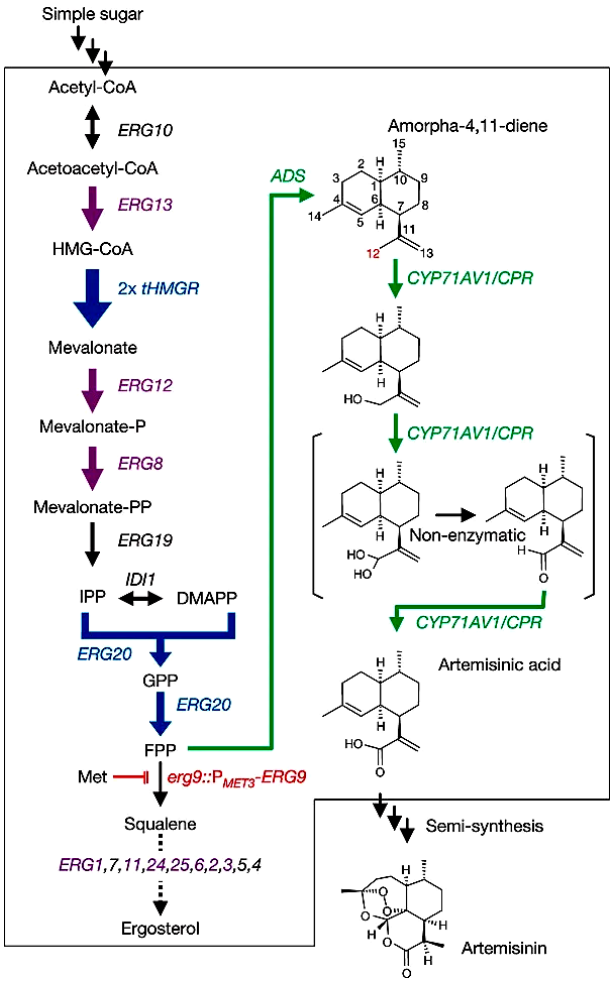


Figure 52 Diagram of the artemisinin production pathway from Ro et al., (2006).

The engineered yeast *S. cerevisiae* strains for the production of taxol and artemisinin clearly provided a possible platform for a higher yield production of the early stage of pleuromutilin biosynthetic pathway in yeast, which is the main focus of this chapter.

5.2 Aims

The aim of the work reported in this chapter was to create the yeast expression vector for the early steps of pleuromutilin biosynthesis pathway and to compare the production efficiency of two different heterologous yeast strains.

Several objectives were set in order to complete the aim:

1. To construct the yeast expression vectors for GGS and Cyclase genes

2. Yeast transformation for the heterologous expression under the control of inducible promoters

3. Chemical analysis to evaluate and compare the yield of the first metabolite extracted from two different engineered yeast strains

5.3 Results

5.3.1 Design and construction of the expression vector pESC-TRP/GC

The YPH499 strain of *S. cerevisiae* is a typical lab yeast strain, which contains non-revertible auxotrophic mutations that can be used for the selection of inserted vectors. The YPH499 strain was used for the heterologous expression of GGS and Cyclase genes from pleuromutilin biosynthesis pathway to accumulate the 3-deoxo-11-dehydroxy-mutillin (metabolite 1), the main precursor of the pathway.

The pESC-TRP vector was used as a backbone vector to construct the expression vector for GGS and Cyclase genes. The pTYGSargGGSCYC plasmid was utilized as a template vector to amplify the GGS and Cyclase genes. The PCR product of GGS was cloned into the *Not*I restricted site of vector pESC-TRP under the control of the *GAL*10 promoter, while, the PCR product of Cyclase was cloned into the *BamH*I restricted site of vector pESC-TRP under the control of the *GAL*1 promoter. The correct construction of the plasmid was checked by PCR for the presence of the genes inserted and by restriction digestion with *Hind*III (Figure 53 and 54).

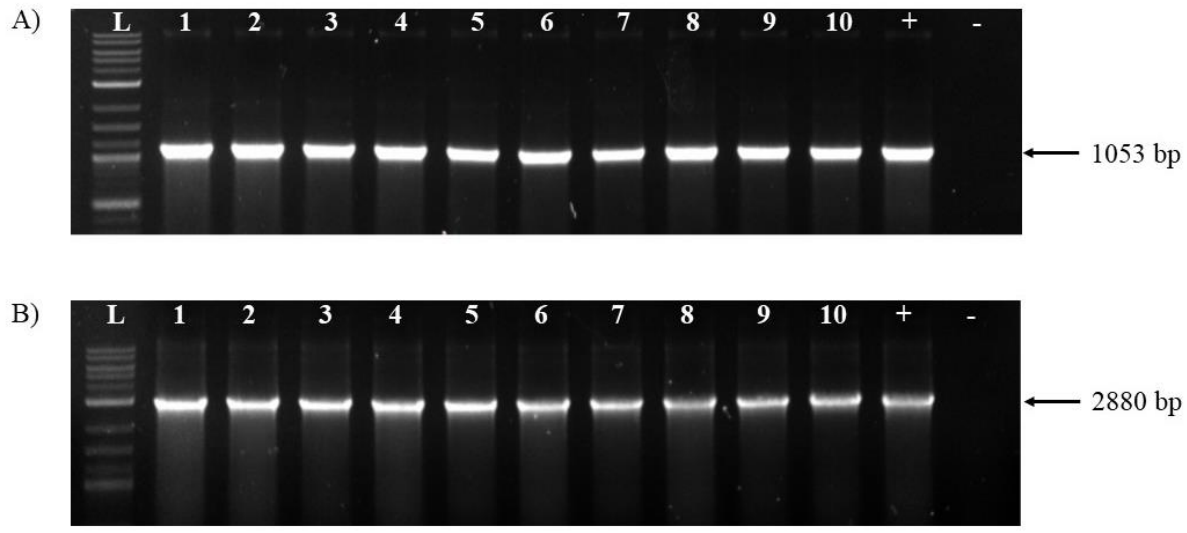


Figure 53 Gel electrophoresis showed the successful PCR amplification of A) GGS and B) Cyclase genes from pESC-TRP/GC expression vector from the *E. coli* transformant colonies.

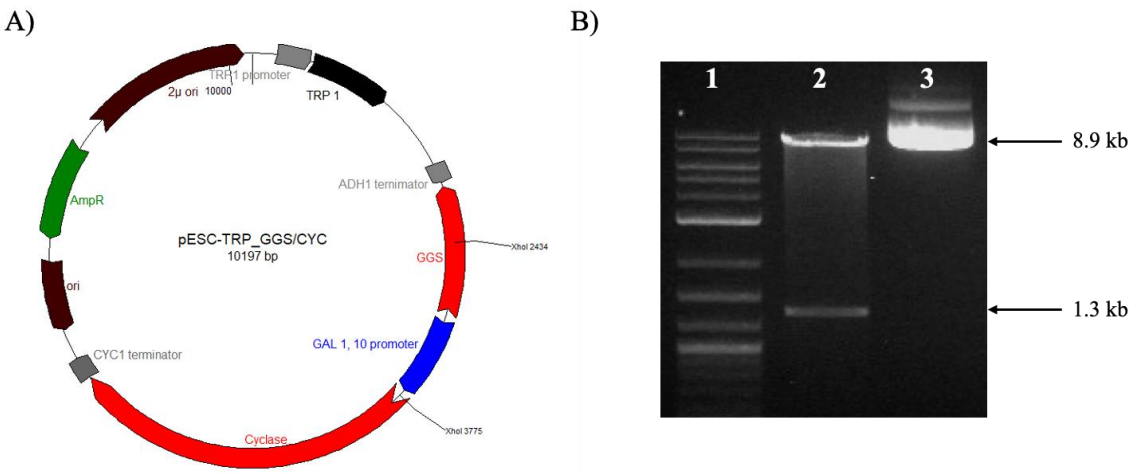


Figure 54 A) The plasmid map of pESC-TRP/GC B) gel electrophoresis of restriction digestion reaction of the pESC-TRP/GC vector with *Xho*I enzyme. Lane 1 contains 5 µL of 2-log ladder. Lane 2 contains 10 µL of digestion reaction with restriction enzyme *Xho*I of expression vector pESC-TRP/GC (expected fragments 8.9 and 1.3 kb). Lane 3 contains 1 µL of undigested plasmid DNA of pESC-TRP/GC.

Having confirmed that the plasmid was properly constructed, pESC-TRP/GC vector was introduced into the yeast YPH499 strain through the yeast transformation procedure following the method described in Chapter 2. The selection of yeast transformant strains was performed on the yeast nitrogen base agar plates without tryptophan, yielding numerous independent transformants.

5.3.1.1 Chemical analysis of 3-deoxo-11-dehydroxy-mutilin in the yeast YPH499 strain

In order to assess the 3-deoxo-11-dehydroxy-mutilin (metabolite 1) accumulation, three independent colonies containing pESC-TRP/GC, named YGC 1, 2 and 3 were subsequently picked and used to inoculate in 5 mL yeast minimal media lacking tryptophan as a starter culture and incubated overnight at 28^{o}C in incubator shaker. On the following day, the starter culture was determined the absorbance at 600 nm, to reach OD_{600} = 1.0. The 1 mL aliquot of these cultures were transferred into the flask contained 50 mL of sterile yeast minimal medium lacking tryptophan and supplemented with galactose, as the genes under the control of *GAL* promoters require galactose for induction. The control flask also contained the same medium but no supplement of galactose. The cultures were analysed for the metabolite 1 production after 6 days of incubation at 28^{o}C.

To evaluate the metabolite 1 production, the cultures were extracted following the procedures in Chapter 2 and analysed on the TLC plate. The results showed that all extracts from yeast transformants YGC 1, 2 and 3 produced the expected metabolite 1 (Figure 55). Especially extract from strains YGC 2 and 3 that showed more accumulation of expected product than the negative control strain (N) containing the empty vector pESC-TRP. Even though the hypothesis was expected no production of any metabolite in negative control strain, but the spots revealed from negative control strain suspected to happen through the contamination during extraction processes. While no accumulation found on the extract from *A. oryzae* strain TR5 containing GGS and Cyclase used as a positive control which might due to the extract was too old.

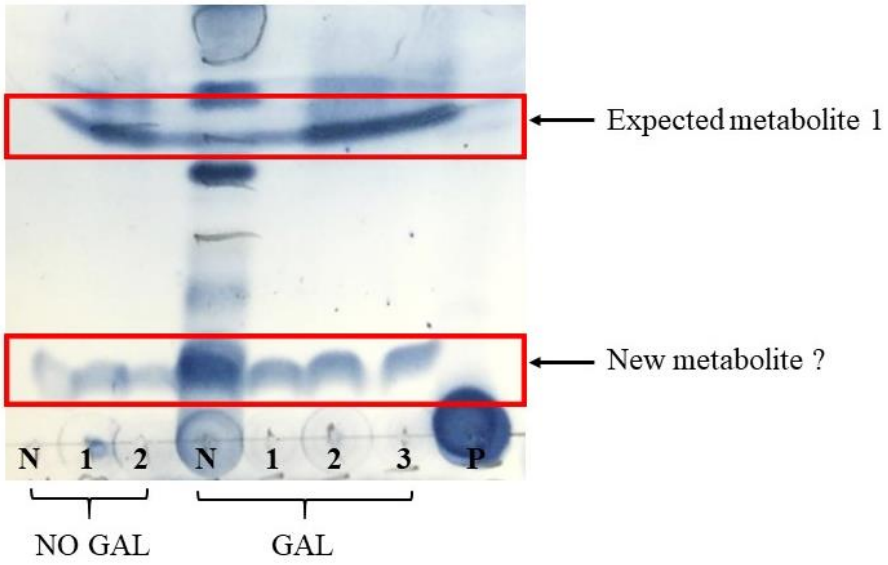


Figure 55 TLC analysis of chemical extracts from yeast strains YGC 1, 2 and 3 harbouring pESC-TRP/GC, grown in two different liquid culture conditions: with and without galactose supplement, compared with N; the YPH499 strain containing empty vector pESC-TRP (as negative control), and P; the crude extract from *A. oryzae* strain TR5 containing GGS and Cyclase genes (as positive control).

5.3.2 Strain construction and chemical analysis of 3-deoxo-11-dehydroxy-mutillin in the engineered AM109 strain

The AM109 strain of *S. cerevisiae* was engineered by focusing on the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), the rate-limiting step enzymes responsible for converting HMG-CoA to mevalonate in MVA pathway. These enzymes are usually sensitive to degradation. Thus, by containing three copies of stable versions of HMGCR, plus the deletions of three genes encode for enzymes that have a degradation effect on HMGCR, resulting to the AM109 strain that was proven to help increase the flux towards terpene precursor (Ignea et al., 2012).

In this project, the hypothesis was that by using the AM109 strain as a host for the expression vector for pESC-TRP/GC, it should gave rise to higher level of metabolite 1 production than the normal YPH499 strain. Yeast transformation and selection of transformant strains was done with the same procedures as in the previous YPH499 strain. The chemical analysis to

determine the production of metabolite 1 was carried out under the same protocol as described earlier. The hexane extract from AM109 transformant strains AGC 1 and 2 containing pESC-TRP/GC vector were visualized through TLC analysis. Unfortunately, the results from TLC plate (Figure 56) showed the similar pattern as appeared in the YPH499 transformant strains. For a better understanding, an attempt to purify and identify the metabolites should be carried out in the future.

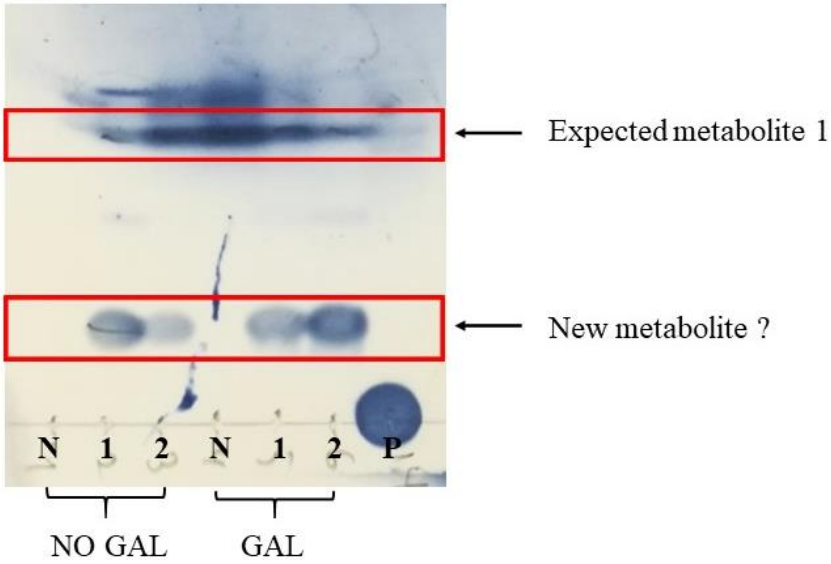


Figure 56 TLC analysis of the crude extracts from yeast strain AGC 1 and 2 containing pESC-TRP/GC, grown in two different liquid culture conditions: with and without galactose supplement, compared with negative control (N); the AM109 strain containing empty vector pESC-TRP and positive control (P); the extract from *A. oryzae* strain TR5 containing GGS and Cyclase genes.

5.4 Discussion

According to the heterologous expression of pleuromutilin biosynthetic pathway in *A. oryzae* and *N. tabacum* host systems, the results from both systems failed to show an increasing of pleuromutilin production. Then, in this chapter yeast-based system platform was chosen as a host for the heterologous expression of the GGS and Cyclase genes. It is anticipated that by increasing the production of the first metabolite from the expression of GGS and Cyclase will lead to increase the flux supply of the bottleneck precursor through the whole pleuromutilin pathway.

Firstly, the yeast strain YPH499 was used for the heterologous expression of pESC-TRP/GC vector. From the TLC analysis, the extract from all YPH499 transformant strains showed accumulation of expected metabolite 1. This suggest a possibility of yeast as a host for the heterologous of the pleuromutilin biosynthesis pathway. However, it worth to note that the negative control strain supposed to not produce any metabolites, but the spots found from this strain might be occurred through the contamination whether during fermentation or extraction processes. Further attempt was done by using engineered *S. cerevisiae* strain AM109, which harboured a set of modified genes that has been proven to increase the production of terpene compounds, as a host for the heterologous expression of pESC-TRP/GC vector by aiming that the production of metabolite 1 should be higher than the YPH499 strains. Unfortunately, the results from TLC analysis from the extracts of AM109 transformant strains 1 and 2 seemed to give low accumulation of metabolite 1 than in the YPH499 strains. However, the spots that obviously found from both YHP499 and AM109 transformant strains could possibly suggested a potential of yeast host to generate a new metabolite.

It is possible that the spots from TLC analysis of the non-induced galactose group could have happened due to too long period of time used to culture yeast cells. This factor could lead to a starving condition for yeast cells. Generally, glucose as a carbon source in the media causes repression of the *GAL* promoters, so the depletion of glucose during starving condition could possibly have implications to the leaky effect of the *GAL* promoters. This suggested that by shorten the culture period to 48-72 hours might be able to prevent this problem. A similar condition was reported in a study in which focusing on the effect of glucose concentration on the *GAL* promoter (Peng et al., 2018). The 48-hour colonies were cultured and collected for fluorescence analysis of yEGFP gene under control of the *GAL* promoter. As a result, the

colonies from the 200 g/L glucose showed higher expression of yEGEP than the colonies from the 20g/L glucose in liquid cultures (Peng et al., 2018).

Previous study from Engels et al., (2008) showed no accumulation of the early part of the taxol biosynthetic pathway using plant GGS and Cyclase genes. But in this study, the results demonstrated the possibility for the early stage of the pleuromutilin pathway production, using fungal GGS and Cyclase genes. Thus, it can be concluded that heterologous expression of fungal genes is more efficient than the plant genes in yeast. This suggests the potential of yeast as a host for further modification of the pleuromutilin biosynthetic pathway in this host system.

Lastly, to construct a complete pleuromutilin biosynthesis pathway in yeast, the host has to go through several rounds of transformation. The genes in pleuromutilin cluster needed to split into several vectors due to the limitation of promoters and selective markers in yeast. Then, at least 10 transformants strains harbouring the desired genes are needed to be screened for a more reliable result. Additionally, analysis for the expression of genes need to be done in order to select a transformant with highly expressed of the genes which have a capacity to accumulate an intermediate before entering another step of the pathway.

5.5 Summary

Heterologous expression of pleuromutilin biosynthetic pathway in yeast has shown to be a potential platform for enhancing the production of the antibiotic drug. In addition, this system also suggested a possibility to generate a new metabolite. However, the carefulness during the fermentation and purification processes have to be noted to prevent a contamination. The attempt to analyse and identify the new metabolite for its antibiotic property have to be further investigated. Moreover, in order to increase pleuromutilin production, the other following genes within the pleuromutilin gene cluster needed to introduce into the yeast cells, following by an analysis of gene expression and determination for the production of desired compounds.

Chapter 6 Discussion

The antibiotic resistance crisis is recognized as a major threat for human health to controlling infectious diseases worldwide. The problem leads to three major effects; higher medicinal cost, prolonged hospital stays and increased mortality. Each year around two million people in the United States are affected by bacteria resistant to antibiotics, with approximately 23,000 individuals dying from these infections (Martens and Demain, 2017), while 25,000 people in Europe are killed by drug-resistant bacteria per year. According to the World Health Organization (WHO), the worldwide healthcare could lose as much as $100 trillion or £65 trillion annually for the treatment of antibiotic resistance (Arias and Murray, 2015). Despite an urgent and serious concern to find new effective drugs to treat infections, only a few new drugs have been developed and introduced into the market since the golden era of antibiotic (Ventola, 2015). To overcome the problem of high bacterial resistance, and no new drugs to treat the infection, renewed research efforts are currently focusing on finding and developing new classes of antibiotic.

Pleuromutilin, a natural-product antibiotic from the fungus *Clitopilus passeckerianus*, has gained a lot of scientific attention for its unique mechanism of action. It showed a strong activity toward multidrug-resistant MRSA strains by inhibiting protein synthesis in bacteria. The drug also has no target-specific cross-resistance to other antibacterials (Paukner and Riedl, 2017). Recent study from Llabani et al., (2019), also reported that chemical modification of pleuromutilin structure leaded to a set of compounds in which biological screening provided a potential to induce the death of cancer cells. These attractive profiles of pleuromutilin make it more appealing for the development of the drug for potential human use. In August 2019, lefamulin (Xenleta^{TM}), a semisynthetic pleuromutilin was successfully developed and approved by FDA to treat Community-acquired bacterial pneumonia in adult. It was the first systemic antibiotic available for both oral and injection forms that has launched into the market over the past twenty years (FDA, 2019). But the commercial cost of the drug is too high, therefore limiting access for number of patients.

Thus, further approaches in the development of pleuromutilin production that would be able to meet the growing demand in the future along with the affordable price are still needed. Heterologous expression systems for the production of pharmaceutical compounds is a

rational strategy to use as an attempt to overcome the difficulties of low production from the original host. It is also providing the opportunity to manipulate the function of genes involved in this biosynthetic pathway more readily or even to generate path for the production of new compounds. Earlier successful cases of drug use in human such as insulin, artemisinin and antibodies also utilised heterologous platforms. The production of insulin for treatment of human diabetic is now being produced mainly in *E. coli* and *Saccharomyces cerevisiae* (Baeshen et al., 2014). Zmapp, a monoclonal antibody that was developed to fight against the outbreak of Ebola virus infection, was also produced in tobacco plants (Yao et al., 2015). Many efforts of partial biosynthesis for the production of artemisinin and taxol, by providing the precursor of the biosynthetic pathway towards the heterologous systems, resulted in increasing titres of the desired metabolites (Fu et al., 2020, Chen et al., 2019). These examples suggest a potential for heterologous systems for improvement of pleuromutilin production. To this content, the aim of this PhD project was to find alternatives and more flexible heterologous systems in order to potentially enhance the yield of pleuromutilin production.

In order to make attempts to enhance and develop the production of pleuromutilin, the basic knowledge of its biosynthetic pathway and the identification of genes involved in each step is required. Fortunately, the previous study has already reported and identified that there are seven genes consisting in the pleuromutilin biosynthetic gene cluster (Bailey et al., 2016). Characterization of the function of each gene, which led to the accumulation of the intermediates along the pathway by heterologous expression, was also done by Alberti et al., (2017). The next challenging step is to choose the most appropriate heterologous host system for the expression of the pleuromutilin gene cluster which could lead to higher production level, then case-by-case analysis of each system is required. Within this PhD project, three different heterologous host system; fungal (*A. oryzae*), plant (*N. tabacum*) and yeast (*S. cerevisiae*) were chosen for the expression of the pleuromutilin gene cluster.

Results from this project suggested the advantages and disadvantages of each heterologous host systems for the attempts to enhance pleuromutilin production and can be concluded in several issues. Undoubtedly, there are some challenges in order to recreate the whole pleuromutilin biosynthesis pathway and perform functional expression of the seven genes involved in the heterologous hosts. The results from this study showed that the plant-based system provided the most appropriate cloning method to reconstruct the expression vector of

pleuromutilin biosynthesis pathway. The Golden Gate cloning kit for plants used in this project providing 96 standard parts that include plant promoters, reporter genes, antigenic tags, selectable markers, and terminators (Engler et al., 2014). This molecular toolkit allowed the seven genes in pleuromutilin biosynthesis pathway to be assembled into one expression vector, even though the efficiency of the cloning was lower from what it claimed from the journal. In this study, successful constructs containing the seven genes of pleuromutilin biosynthesis pathway, which was used to transform with just one round into the tobacco plant, showed the advantage in term of time scale of transformation process when compared to the fungal and yeast system. In case of the yeast host, the availability of the cloning sites became a limiting factor. In this project, the problem was encountered when using the yeast vector pESC-TRP which limited the insertion of genes to only two. The remaining genes must split into another vectors, which means that several rounds of transformation are required. Similar limitations also occurred in fungal-based systems; the fungal vector pTYGSarg, pTYGSade and pTYGSbar utilised in the study permitted the insertion up to three genes for each vector and at least three rounds of transformation were needed. Co-transformation of two expression vectors might be a strategy to solve the problem. However, the result from this strategy could lead to the lower efficiency of the genes insertion when compare to the normal transformation procedure of one round one expression plasmid. The study from Alberti et al., (2017) showed that the efficiency of the insertion of transgenes from the transformation of one plasmid per round in *A. oryzae* range from 60% to 75%, while the result from co-transformation of two vectors decreased the efficiency of the insertion down to 20%.

Apart from the challenge of the construction of the expression vector, the potential of the hosts to enhance pleuromutilin production was considered. According to the results from this project, yeast *S. cerevisiae* has been shown to be the most favourable platform for the development and pathway modification for the higher production of pleuromutilin, even though unexpected results were revealed from the chemical analysis of the yeast transformant strains. Within the time limit of this project, only preliminary approaches could be conducted in the yeast system. There are more investigations that should be done in this microbial platform. The successful cases of the metabolic engineering for the production of the intermediates of artemisinin and taxol as previously described in Chapter 5 could be used as a guideline for the metabolic engineering of pleuromutilin production. Furthermore, the study from Paddon et al., (2013) also reported the successful expression of the complete pathway

for artemisinic acid production. The *S. cerevisiae* strains were engineered for the higher production of artemisinic acid by optimising the expression ratio of CYP71AV1:CPR1 and combining with the expression of other three genes derived from *A. annua* (CYB5, ADH1 and ALDH1), resulting in more than ten-fold increase of artemisinic acid titres or 25 g/L of the fermentation batch. In addition, they also developed the powerful, efficient and scalable chemical synthesis process in order to convert artemisinic acid to artemisinin. Compared to other methods they utilised singlet oxygen as a chemical source, screened several catalysts and provided the most efficient one into the reaction and also employed telescoping to the reactions to prevent any exhaustive of intermediates during purification and isolation. They have proved that by applying these chemical synthesis procedures with the 2 reactors of industrial batch reactor (5,000 – 10,000 L) resulted in the artemisinin production around 0.5-1.0 tons per day.

Plant-based systems provide effective heterologous expression especially for the production of valuable medicinal protein products. Factors that make plant systems favourable for recombinant protein production include: high value of pharmaceutical protein products, low-cost biomass production compared with microbial fermentation, the potential for large-scale production using agriculture, the low risk contamination of products and no downstream process for protein purification is required for edible proteins produce in plant food (Doran, 2006). The examples include an IgA/G antibody against oral streptococcal colonisation (Ma et al., 1998), an edible vaccine against an *E. coli* enterotoxin in transgenic potato (Tacket et al., 1998), an edible vaccine against hepatitis B in transgenic lupin and lettuce (Kapusta et al., 1999). However, the metabolic engineering of plant for natural secondary metabolites production is still limited. The reasons might due to the structural complexity of the targeted compound, lack of knowledge of how to optimise the expression of heterologous genes, less understanding of expected metabolic networks within the native *in planta* biosynthesis, high competition of other metabolite pathways and desired compounds purification problem (Ikram et al., 2015).

The well-established strategy to enhance the production of heterologous terpenoid compounds in plants refer to the cases of artemisinin biosynthesis and taxol biosynthesis. But it must be noted that those approaches include only a limited number of steps in biosynthesis. In both cases only partial heterologous expression of the genes involved in the pathway was successfully done. It is also the heterologous expression of plant genes to another plants. In

addition, the overall yield obtains from heterologous production resulting in the range of µg/L to mg/L from shake-flask culture. When considered the biosynthesis of pleuromutilin which involved multiple steps of enzymes activity and including the fact it is the heterologous production of the cross kingdom, so the metabolic engineering of pleuromutilin biosynthesis pathway will be more complicated and time-consuming to optimize *in planta*. However, the results from this project revealed the small trace of expected metabolite 2 from HPLC analysis. This might be suggested a hopeful possibility for further challenges to modify and develop the heterologous expression of pleuromutilin *in planta*. Furthermore, the result could be pinpointed as a stepstone for the heterologous expression of other fungal metabolites to be performed *in planta*.

It is also worth noting that to synthesize a complex diterpene structure such as pleuromutilin, the expression of multiple CYP 450s are needed. Thus, to optimize the CYP 450s in order to fully function in the heterologous host, yeast and tobacco, is totally a challenging approach. These enzymes play a critical role for the pleuromutilin scaffold modification. Due to their nature as heme-containing enzymes, most CYP required its redox partner, cytochrome P450 reductase (CPR), to transfer the electrons from NADPH for the activation of oxygen in the P450 catalytic cycle (Ikram et al., 2015, Xiao et al., 2019). Lack of electron transfer from CPR to CYP may result in the low activity of CYP, thus the CPR partner usually require for the heterologous expression along with CYP to support its activity (Xiao et al., 2019). Previous study from Zhang et al., (2011) reported that the heterologous expression of the three genes from *A. annua,* ADS, CYP71AV1 and DBR2 in tobacco results in the accumulation of dihydroartemisinic aldehyde but no artemisinic acid, the final intermediate of artemisinin pathway was detected. This result was not in agreement with the heterologous expression in yeast by Ro et al., (2006). The study from Ro et al., (2006), the heterologous expression of the genes derived from *A.* annua ADS, CYP71AV1 and its redox partner CPR, resulted in the production of 32 ± 13 mg/L artemisinic acid from liquid culture. The different results from these two studies suggest that the co-expression of CYP and its redox partner is significantly increase in its activity for further terpenoid structure modification.

For this study *A. oryzae* seemed to be the less suitable heterologous host for pleuromutilin production. According to the attempts to overexpression the genes under a strong promoter, the results from this study showed no improvement in the functional expression of the genes. Even though, *A. oryzae* has the benefit in term of the ability to obtain high number copies of

expression vectors of the pleuromutilin biosynthesis gene cluster which could lead to higher titre of desired compounds. While yeast and plants would not be affected by the number of copies of the expression vectors. But many drawbacks such as the time-consuming techniques for the transformation and fermentation processes and instability of the transformant strains that could possibly happened during the strains selection, make this system not attractive for improving pleuromutilin production.

Attempts for detection and purification of the intermediates from pleuromutilin biosynthesis pathway are the final step to investigate the improvement for pleuromutilin production. Within the three hosts, yeast might be the easiest system to manage for theses production processes. It offers a short time fermentation and including several steps for purification. Moreover, yeast mainly produce sterol, so there is less interference from the other secondary metabolite pathways. Whilst tobacco plants look like the most difficult system to purify compounds, the system is known to be a rich source of compounds and there are many native secondary metabolite pathways. Thus, metabolic engineering to decrease the interference from other competitive pathways and purify the desired compounds is seemed to be complicate procedures. The fungal host seems to have major disadvantages because of the time-consuming processes of strains construction until it reaches to the fermentation for mass production.

As a conclusion, the results from this thesis have evaluated the potential of the three heterologous expression systems as rational platforms to enhance pleuromutilin production. It has also suggested a potential for the production of new compounds within the heterologous host. Yeast seems to be the most capable system for the metabolic engineering of pleuromutilin biosynthesis pathway. This because of its well-established platform and accessible knowledge for heterologous expression and a large-scale production from other terpenoids. Plant-based systems need to be carefully considered for the long-term project for the metabolic engineering until it could search the industrial scale and the obstacle of the purification process. Whilst the fungal host system seems to be the less attractive of hosts tested. The system faced the limitation for the improvement for the expression of the transgenes and the great drawback in term of time-consuming when compared to other systems. The outcomes from this study has demonstrated the potential to engineer heterologous hosts for improving the targeted pleuromutilin production and could be used as

a stepstone for the future attempts in order to develop metabolic engineering of pleuromutilin or other valuable metabolites.

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